Graduate School Form 30
Updated 1/15/2015

PURDUE UNIVERSITY
GRADUATE SCHOOL
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By Keyin Lu

Entitled

ISCHEMIC PRECONDITIONING AND HYDRODYNAMIC DELIVERY FOR THE PREVENTION OF ACUTE KIDNEY INJURY

For the degree of Master of Science

Is approved by the final examining committee:

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Approved by: Simon J. Atkinson 7/16/2015

Head of the Departmental Graduate Program Date

ISCHEMIC PRECONDITIONING AND HYDRODYNAMIC DELIVERY FOR THE PREVENTION OF ACUTE KIDNEY INJURY

A Thesis

Submitted to the Faculty

of

Purdue University

by

Keyin Lu

In Partial Fulfillment of the

Requirements for the Degree

of

Master of Science

August 2015

Purdue University

Indianapolis, Indiana

I dedicate this thesis to my mentors, family and friends for their unconditional support and guidance in this incredibly rewarding journey for knowledge.

ACKNOWLEDGEMENTS

I would like to thank my thesis advisor, Dr. Simon Atkinson, for his advice and encouragement in support of my research and studies. I could not have asked for a better advisor to advise, motivate and challenge me through this process. I have learnt so much from him, and yet so much more to learn from him.

I would also like to express my immense gratitude to Dr. Robert Bacallao. He gave generously of his time and knowledge, giving advice, answering questions and challenging me to delve deeper into the data. I will always carry his stories and words of wisdom with me, especially this one “Hope is not a protocol”. His passion and excitement for research and finding answers to problems is simply contagious and it has been such a pleasure learning from him.

I would also like to thank Dr. Bonnie Blazer-Yost for her encouragement and suggestions. She lends a critical eye that pushes me to strive for better and better results and analysis. My gratitude goes out to Dr. George Rhodes, for training me in animal surgery and assisting me in troubleshooting the most problematic aspects of conducting animal research.

Our interaction was short, but Dr. Peter Corridon has laid the immense foundation upon which my thesis is built. His ingenuity lives on in our research. Dr. Mark Hallett has also been extremely helpful and patient in guiding me through my research. I would also like to thank Weimin Xu, who set an exceptional benchmark for diligence and technical skills in a researcher.

Last but not least, I thank my lab partners Shijun Zhang and Alexander Kolb for hustling with me through all the surgeries, mitochondria assays, the early mornings, the late nights and all the other crazy and non-crazy times. They were my sounding board for ideas, and such incredible teammates in our Kidney Juicers team. I look forward to all the amazing things that we will accomplish in our futures as researchers and medical professionals.

TABLE OF CONTENTS

Page

Page

Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

Figure Page

ABSTRACT

Lu, Keyin. M.S., Purdue University, August 2015. Ischemic Preconditioning and Hydrodynamic Delivery for the Prevention of Acute Kidney Injury. Major Professor: Simon J. Atkinson.

Acute Kidney Injury (AKI) is a prevalent and significant problem whose primary treatment is supportive care. Ischemic preconditioning is a strategy used to protect organs from ischemic injury via a prior injury. Ischemic preconditioning in the kidneys has been shown to confer protection onto kidneys from subsequent ischemic insults with attenuated serum creatinine values in treated rats. In the preconditioned kidneys, the enzyme IDH2 was discovered to be upregulated in the mitochondria. Hydrodynamic fluid delivery to the kidney was found to be a viable technique for delivering this gene to the kidney, resulting in artificially upregulated expression of IDH2. Via a two-pronged effort to discern the functional significance of ischemic preconditioning and hydrodynamic IDH2 fluid injections, we performed mitochondrial oxygen respiration assays on both preconditioned and injected kidneys. We found that renal ischemic preconditioning resulted in no significant difference between sham and preconditioned, subsequently injured kidneys, which is similar to the results from the serum creatinine studies. Hydrodynamically IDH2-injected, and subsequently injured kidneys respire significantly better than vehicle injected, and subsequently injured kidneys, which shows that hydrodynamic injections of IDH2 protects kidneys against injury, and partially mimics the effects of preconditioning.

CHAPTER 1. INTRODUCTION

1.1. Acute Kidney Injury

1.1.1 AKI: A Significant and Common Clinical Problem

Acute kidney injury (AKI) refers to a common and significant clinical syndrome that is characterized by a rapid decrease in renal function, leading to an accumulation of blood urea nitrogen, creatinine and other waste products. The term AKI has replaced acute renal failure (ARF) used to in older literature to describe a spectrum of clinical manifestations that presents acutely in the kidney, with accompanying increasing mortality and worse prognosis associated with increasing serum creatinine values. AKI is common in hospital patients, especially in critically ill patients, whose AKI is often secondary to extrarenal events[1]. There are more than 5000 cases per million people per year for non-dialysis requiring AKI and 295 cases per million for dialysis-requiring manifestations[2]. AKI is especially common in critically ill patients with an occurrence of more than 36% after admission to an ICU, with septic AKI prevalent 24 hours after admission[3, 4].

AKI is a great healthcare concern because of its high prevalence and substantial treatment cost. In 2011, AKI costs about $4.7 billion for approximately 498,000 hospital stays[5]. Even with new prevention and support strategies, AKI has high morbidity and mortality rates. The ICU population is at immense risk due to multi-organ disease and sepsis[3, 6, 7]. There are no direct and effective targeted therapies for AKI and

treatment is mostly supportive. However, new diagnostic techniques in the form of renal biomarkers may help with early detection and accelerated recovery. Patients with past history of AKI are also at increased risk of chronic kidney disease[1].

1.1.2 Causes of AKI

AKI can be caused by sepsis, cardiac shock, disease, crush injury, contrast agents and antibiotics.[8-14]. The mechanism of injury by these agents still remains to be elucidated. From a pathogenic standpoint, the causes of AKI are conventionally grouped into prerenal, intrinsic and postrenal[15].

Prerenal AKI is the most common cause of AKI in hospital inpatients and critically ill patients, with higher prevalence in developed countries[16]. Prerenal AKI occurs when there is renal ischemia, a lack of blood flow to the kidney. Renal artery stenosis[17, 18], renal vein thrombosis [19], low blood volume, low blood pressure, heart failure, liver cirrhosis, exposure to substances harmful to the kidney, inflammatory process in the kidney are all potential causes of prerenal AKI[20].

Intrinsic AKI occurs when there is injury intrinsic to the kidney. The injury can be further categorized based on the structure affected. There can be damage to the glomeruli resulting in glomerulonephritis, tubules resulting in acute tubular necrosis (ATN) or renal interstitium resulting in acute interstitial nephritis[21].

Postrenal AKI occurs when there is obstruction in the excretion of urine. This may be cause by urinary tract obstruction, prostate problems, kidney stones, obstructed urinary catheter or tumor masses in the bladder or urethra.

Some causes of AKI are more frequent in certain geographical locations. For example, in developing countries, hypervolemia secondary to diarrhea is a prevalent

cause, whereas in developed countries, hypervolemia secondary to open heart surgery is more common. Also, there are also differences within a country, where certain disorders may have different rates of occurrence in the community and in hospitals[1, 22].

1.1.3 Classification of AKI

AKI has recently replaced the term acute renal failure (ARF) to better depict the spectrum of AKI by the Acute Dialysis Quality Initiative. The system RIFLE (risk, injury, failure, loss, end stage) criteria was developed and supported by the Acute Kidney Network[4, 23-25]. The system criteria has been tried and tested in thousands of patients.

Table 1: RIFLE Criteria for Acute Kidney Injury [4]

	GFR Criteria	Urine Output Criteria
Risk	1.5 Fold increase in sCR or GFR decrease >25%	UO <0.5mL/kg/h for 6h
Injury	2 Fold increase in sCR or GFR decrease >50%	UO <0.5mL/kg/h for 12h
Failure	3 Fold increase in sCR or GFR decrease of >75%, sCR>4mg/dl or acute rise in sCR>0.5mg/dl	UO<0.3mL/kg/h for 24 h or anuria for 12h
Loss	Complete Loss of kidney function >4 weeks	
ESKD	End-stage kidney disease (>3 months)	

The RIFLE criteria show the parameters for classifying a patient's condition from class R (risk) to class F (failure). Class R has a high sensitivity and class F has a high specificity for AKI. sCR – serum creatinine concentration. UO – urine output. GFR – glomerular filtration rate. ESKD – end-stage kidney disease.

1.1.4 Signs and Symptoms of AKI

Since the kidney functions to maintain homeostasis via excretion of waste products of metabolism as well as other regulatory functions, the symptoms of AKI are caused by the accumulation of urea and other nitrogenous waste products that cannot be properly excreted. Symptoms include fatigue, loss of appetite, headache and nausea. There can also be a significant increase in serum potassium that disrupts cardiac function. Due to inability to excrete sufficient fluid from the body, fluid balance is affected, causing edema, or the accumulation of fluid, particularly in the lungs and distal regions of the limbs[26-28].

AKI is diagnosed by simple laboratory tests, with increases in blood urea nitrogen and serum creatinine[29]. Observations of oliguria, or insufficient production of urine, warrant a diagnosis of AKI as well. However, these clinical parameters are relatively late markers in the injury process, as AKI is relatively asymptomatic till late in the injury process, and recent efforts are made to identify new biomarkers and tests that would allow for much earlier detection[30].

In various studies, investigators have identified novel biomarkers for identifying AKI, and the concentrations of these biomarkers seem to change earlier than serum creatinine (sCR) concentrations and can possibly allow for earlier detection in order to administer adequate therapy. These new biomarkers may be able to warn of impending AKI earlier. Different biomarkers also show different aspects of renal segment injury. Cystatin C concentrations seemed to predict changes in glomerular filtration rate and neutrophil gelatinase-associated lipocalin (NGAL) levels are shown to associate with tubular stress and injury[31-40].

1.1.5 Present Management of AKI

Current treatment of AKI is primarily focused on supportive care. Therefore, improved treatment and management is dependent on a better understanding of its underlying causes. Currently, management of AKI is to remove the primary cause (e.g. mediating cardiac output or treating sepsis[13, 18]) and utilize supportive measures to maintain homeostasis while allowing the kidney to recover. There is supporting evidence for starting general nutritional support of adequate calories, vitamins, trace elements and protein early, and specific renal nutritional solutions are not necessary[41].

In patients with hyperkalemia, there are the options of administering insulin, dextrose, bicarbonate (if there is acidosis as well), nebulized salbutamol or calcium gluconate solution. These treatments utilize different ways to induce cellular uptake of potassium: insulin administered with glucose facilitates the uptake of glucose into the cell, and potassium follows as such; bicarbonate increases the extracellular pH, which results in cellular uptake of potassium as well; calcium gluconate stabilizes the cardiac cell membrane against the excitability of the hyperkalemic environment; and sabultamol indirectly stimulates the NaK ATP pump via adenylate cyclase to increase potassium uptake into the cell. Drug therapy should be modified to take in to consideration the decreased ability of the kidney to clear the substance. If there is fluid overload, the use of loop diuretics in patients with polyuria is an option. There are no specific recommendations for fluid management, but renal replacement therapy is recommended at an early stage for critically ill patients [1].

In sum, there are no targeted therapies that can lessen AKI or speed recovery. Fluid, electrolyte and acid-based balance management is mainly supportive treatment option and beyond these measures, in the long term, kidney transplant is an option for patients suffering from irreversible kidney injury and failure[42].

1.1.6 Structure and Organization of Function in the Kidney

In order to understand function and subsequently dysfunction in AKI, it is important to understand the underlying structure and organization of function in the kidney. The unique spatial separation of various transporters, beset in various environments results in the filtration machine that is the kidney. Blood is first filtered through the glomerulus into the Bowman's capsule before entering the proximal tubule. Proximal tubules are the workhorses of the nephron in that they reabsorb glucose, amino acids, bicarbonate, and water back into the cells to reconstitute blood. The driving force is secondary active transport, requiring a high concentration of mitochondria in order to power these processes. The proximal tubules are then followed by the loops of Henle, which are situated in the medulla of the kidney. The loop of Henle ascends to the distal convoluted tubules, which are situated in the cortex along with the proximal convoluted tubule. The cortex region of the kidney has a dense capillary network and a high effective blood flow[43].

Scanning electron microscopy and transmission electron microscopy of kidney sections has been useful in visualizing the unique structures of kidney tissues. In the proximal tubule, the apical regions of cells appear rounded and interlocked, similar to a ball and socket type of interlocking. There is a high density of mitochondria visualized within the proximal tubule cells. The lumens are framed by the brush border, which may appear as craters on the EM sections. Primary cilia are projected above the brush border into the lumen. Vessels are also frequently identified in a proximal tubule section. The distal tubule is difficult to identify, but typically the lumen contains a short microvilli of irregular shape and lacks a brush border[44].

An ischemic event results in significant alterations in morphology of the normal renal tissue, namely disruption and loss of proximal tubule brush border, sloughing off of tubule cells, proximal tubular dilation and distal tubular casts, and areas of cellular regeneration[15, 45, 46].

1.1.7 Pathophysiology and Systemic Responses to AKI

The pathophysiology of prerenal AKI has been studied in animals[47, 48]. In studies occluding the blood flow in the renal artery, there are many pathways that are implicated in the system of organ injury[49, 50]. Coagulation is activated[51], leucocytes are recruited[52], the endothelium is injured[53], adhesion molecules are expressed[54], cytokines are release[55], toll-like receptors are induced[56], intrarenal vasoconstrictor pathways are activated[57] and apoptosis is induced[49, 50]. Tubular cells lose or inverse their polarity[58] and lose adhesion to the basement membrane[49, 50].

Prerenal mechanisms are one of the most common causes of AKI. Due to the high volume of blood flow and the high level of metabolic activity required to process the volume of blood, the kidney is extremely sensitive to injury and toxins. In a renal ischemia/reperfusion injury (IRI), a lack of oxygen delivery and nutrients to the cells of the kidney is combined with a failure to remove waste products[59-61]. Proximal tubules are especially sensitive to hypoxia because they principally depend on oxidative catabolism, whereas distal tubular segments and medullary thick ascending limbs can utilize glycolysis to endure severe hypoxia[62].

Chief regulators of renal regional perfusion and tubular transport activity include nitric oxide (NO), adenosine, dopamine and vasodilator prostaglandins. NO, dopamine and PGE2 enhance blood flow and suppress transport and oxygen consumption while adenosine and other vasoconstrictors reduce cortical blood flow and glomerular filtration rate (GFR), leading to decreases in tubular transport, oxygen consumption and downstream solute delivery.[63, 64]

Morphologically, acute sublethal changes in rat models include the loss of brush border, cell and mitochondrial swelling, cytoplasmic vacuolization and nuclear pyknosis[65]. During a response to ischemia, epithelial cells lose normal cytoskeletal

organization and cell polarity. In the kidney, integrins with B1 subunit are the most common and localize to basal surfaces of tubular epithelia, where they interact with the extracellular matrix of basement membrane. In one rat study with a 40min unilateral ischemia, B1 integrins appear on lateral borders in the epithelial cells of the S3 segment but not on the apical surfaces immediately after reperfusion. As reperfusion continues, epithelial cells attached to the basement membrane localize B1 to the basal surfaces while the polarity of other marker proteins are lost. Sloughing off of cells in the lumen is a common phenomenon during injury and B1 integrins were not detected on exfoliated cells, indicating that B1 integrin distribution is dramatically changed during ischemic injury[58]. Further injury may lead to cell death by either necrosis or apoptosis[66, 67]. Also, in response to injury, the epithelium induces an inflammatory reaction, activating cytokines and vasoactive mediators, which exacerbate vasoconstriction and the inflammatory process.

Endothelial cells are also involved in the kidney's response to AKI. First, damaged endothelial cells contribute to vasoconstriction by decreasing production of nitric oxide and other vasodilators. Small vessels can also be occluded due to the coagulation triggered by the immune response and lead to regional ischemia[53]. Second, damage to the endothelial cell layer increases the permeability of the microvascular barrier and reduces the number of microvessels in the outer medulla, which results in chronic hypoxia even when the injury is resolved[68, 69]. Chronic hypoxia results when tissues are gradually deprived of oxygen, leading to progressive fibrosis, further altering the structure and function of the kidney. There is less delivery of oxygen and nutrients, further epithelial cell injury and further fibrosis, leading to a vicious cycle of injury[70, 71].

There are both innate and adaptive immune responses in ischemic injury. The innate immune response is seen in an early response to injury in a non-antigen-specific mode[72]. Damaged tissue releases endogenous ligands, which upregulate Toll-Like

receptors (TLRs), which activates mature dendritic cells (DCs)[56, 73, 74]. DCs then in turn activate T-lymphocytes, initiating an adaptive immune response. Injured tubular epithelial cells also express adhesion molecules [75] and other ligands for T cells in addition to TLRs[76], and these activate immune cells, leading to further inflammation. Cytokines and chemokines are also released by the epithelium to recruit more immune cells[77]. Various immune cells including monocytes/macrophages, DCs, and T cells, releases factors that allows for increased vascular permeability, thus impairing tubular epithelial and endothelial cells, further worsening the injury[78].

The cellular adaptations and responses to kidney injury and repair are still not yet fully understood and elucidated. Better understanding and further research is needed to improve therapies to prevent renal injury, mediate the recovery process in the event of an injury and minimize the effects of chronic kidney disease.

During AKI, the sympathetic system and neurohormonal responses are activated. The renin-angiotensin-aldosterone system, renal sympathetic system and tubuloglomerular feedback systems are activated, events that signal a general body response to AKI[13, 79, 80]. Direct infusions of norepinephrine into the renal artery resulted in prolonged reductions in blood flow to the kidney similar to AKI[81]. Increased sympathetic innervation also results in the activation of the renin-angiotensin-aldosterone pathway, with elevated renin levels in AKI patients[82].

1.2 Model of Renal Injury

1.2.1 Ischemic Preconditioning

Ischemic preconditioning (IP) is a phenomenon first described by Murry and colleagues on the dog heart in 1986[83]. IP refers to a sublethal ischemia/reperfusion injury (IRI) to an organ that allows the organ to be more resistant to subsequent bouts of injury. The protection conferred by IRI was also demonstrated in other animal models including rabbits, guinea pigs and rats [84, 85] as well as different organs including the heart[85], liver[86], lung[87] and kidney[88].

Although IP was first described in the heart due to its relationship to myocardial infarctions, there is now substantial research supporting the finding that IP can confer protection to the kidney against subsequent bouts of injury[89]. Kidneys are extremely susceptible to ischemia/reperfusion due to its extensive blood flow and workload, and there are extensive cellular responses to resulting hypoxia and repair mechanisms[62]. Animal models have confirmed both local and distant preconditioning effects in the protection of the kidneys[90, 91].

Meta-analysis has confirmed that IP can reduce the parameters of injury in decreased serum creatinine and blood urea nitrogen, as well as structural alterations as observed by histology after IRI compared to controls. This suggests that IP effectively alleviates renal damage after IRI, however the mechanism to the preconditioning phenomenon is yet to be understood. Thus, it is important to understand the mechanisms involved in ischemic preconditioning in the kidney in order to devise therapy that mimics the protective mechanisms of IP, as IP is not a possible clinical approach. Currently, there is evidence that supports two theories for the protective effect of IP in other organs: the opening of mitochondrial ATP-sensitive potassium (mitoKATP) channels and the regulatory functions of protein kinase C (PKC)[92, 93].

In a cellular energy crisis similar to an IP event, mitoKATP channels open and close in order to restore proper membrane potential, internal Ca^{2+} concentration and the degree of membrane swelling. The maintenance of membrane potential allows the mitochondria to maintain the proton gradient for mitochondria ATP synthesis in order to meet the energy demands of the cells[94]. There are three major hypotheses for the mechanism of protection via the mitoK_{ATP} channel. First, mitoK_{ATP} channel openings may attenuate Ca^{2+} ion accumulation, through a partial depolarization in response to the opening of the channel, during ischemia and studies that induced a decrease in mitochondrial calcium ion loading under anoxic conditions resulted in protection of isolated mitochondria[95].

The second hypothesis points to mitochondrial matrix swelling and changes in ATP synthesis as a potential mechanism behind the protection of mitochondria function, as mitochondria swelling is an indication of mitoK_{ATP} channels opening. In a study by Lim et al., preconditioning increased matrix volume by 58% with preconditioning in isolated mitochondria[96]. It is proposed that mitochondria swelling allows the inner and outer mitochondrial membranes to come closer together, so that the membrane and channel proteins on both membranes can optimize contact, resulting in increase in ADP transport and ATP synthesis[97].

A third hypothesis points to the role that mitoK_{ATP} channel plays in apoptosis. Studies involving diazoxide pretreatment of myocardial mitochondria resulted in reduced markers of apoptosis from IRI and mitoK_{ATP} protects against the early stages of apoptosis and protected the integrity of mitochondria[98-100].

Also, MitoKATP is believed to be regulated by several pathways via PKC[101]. There are theories outlining the possible mechanisms, including controlling the release of oxygen radicals and levels of Pcl-2 proteins, a major regulator of apoptosis[102].

1.3 The Role of Mitochondria in AKI

1.3.1 The Function and Structure of Mitochondria

The mitochondria play essential roles in cell survival, both in the form of ATP synthesis and the maintenance of calcium homeostasis. The mitochondrion functions as an energy generator for the cell, producing most of the cell's supply of ATP, but is also involved in signaling, cell growth and cell death[103]. The mitochondria play important roles in metabolism, metal ion homeostasis and are major sources and regulators of reactive oxygen species (ROS)[104].

The mitochondrion is unique in terms of its structure in the cell; it consists of an outer and inner membrane. The two membranes differ in permeability and composition, and help establish a unique environment between the membranes that allows for ATP synthesis. The outer membrane is embedded with pores that allows target proteins and ions to pass while the inner membrane is less permeable to ions and smaller proteins, and embedded with proteins involved in the electron transport chain (ETC) and ATP synthesis. The inner membrane encloses the mitochondrial matrix, which contains enzymes involved in the citric acid cycle, the pyruvate dehydrogenase complex, fatty acid oxidation enzymes, amino acid oxidation enzymes and other soluble metabolic intermediates[105].

In the citric acid cycle (Figure 1), pyruvate is imported into the mitochondrial matrix through a pyruvate protein carrier. Pyruvate first undergoes oxidative decarboxylation, catalyzed by pyruvate dehydrogenase complex (PDH) to produce acetyl CoA, where electrons are withdrawn to make ATP and carbon dioxide is released. Next, acetyl CoA enters the citric acid cycle when it combines with oxaloacetate to generate citrate via thioester hydrolysis with the enzyme citrate synthase. Citrate is converted in the next step into isocitrate, a better substrate for oxidation via aconitase. Isocitrate is

then oxidized by isocitrate dehydrogenase in an oxidative decarboxylation reaction into alpha-ketoglutarate, a 5-carbon intermediate, producing NADH and is fed into complex I of the electron transport chain (Figure 2). Alpha-ketoglutarate is further undergoes oxidative decarboxylation to become succinyl-CoA via the alpha-ketoglutarate dehydrogenase complex. Succinyl-CoA then undergoes thioester cleavage to generate GTP and succinate. Succinate is then oxidized by succinate dehydrogenase to fumarate, with electrons accepted by FAD, $FADH_2$ is subsequently fed into complex II of the electron transport chain (Figure 2). Succinate dehydrogenase is the only enzyme of the citric acid cycle not found in the matrix, but tightly bound to the inner mitochondrial membrane in order for electrons to be passed directly from $FADH_2$ to Coenzyme Q in the electron transport chain (ETC) [105].

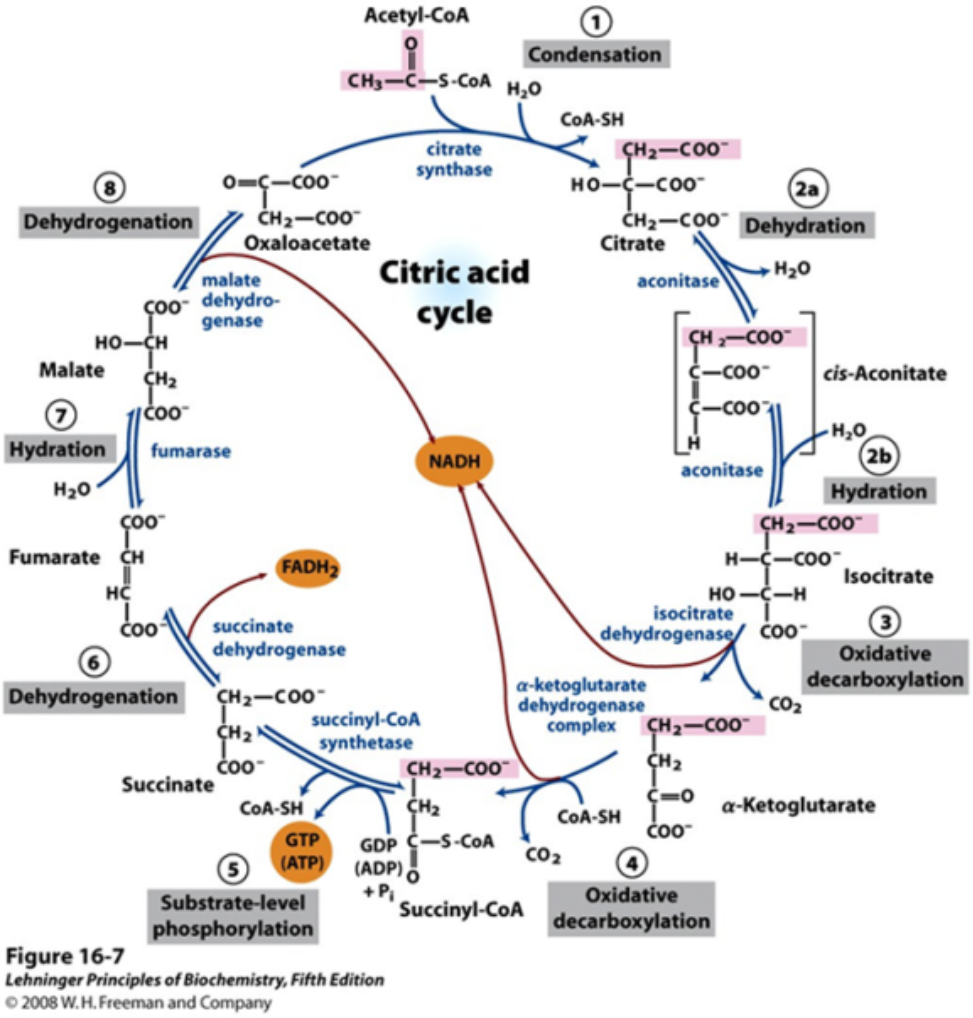

Figure 1: The Citric Acid Cycle (TCA) [105]

During the Citric Acid cycle, electrons are harvested to reduce cofactors flavin adenine dinucleotide ($FADH_2$) and nicotinamide adenine dinucleotide (NADH), and these electrons enter the electron transport chain in the inner mitochondrion membrane to produce energy in the form of ATP. This process, oxidative phosphorylation, is achieved by the movement of electrons along four complexes in the electron transport chain. Complex I, ubiquinone oxidoreductase, accepts two electrons from NADH, converting NADH to NAD+ and at the same time, pumps hydrogen ions from the matrix into the inner membrane space. Complex II, succinate dehydrogenase and the only enzyme of

the citric acid cycle not found in the matrix but in the mitochondrial membrane, accepts electrons from $FADH_2$ in order to reduce cytochrome c, or complex III, and more hydrogen ions are pumped from the matrix into the inner mitochondrion space. Complex IV, cytochrome oxidase, passes the electrons along the chain to the final electron acceptor, oxygen, and more hydrogen ions are pumped. The generation of this electrochemical gradient across the membrane due to the hydrogen ions is used to power complex V, ATP synthase to produce ATP from ADP[105].

The interdependence of proteins embedded in the membranes coupled to the chemical processes results in a dynamic and fluid environment, which is crucial for mitochondrial function. The membrane impermeability of the inner membrane to protons establishes the proton gradient, which is crucial to the function of ATP synthase[106]. The fluidity of the membrane is important to organizing the ETC complexes and when the optimal membrane fluidity changes, mitochondrial functions can be affected[107, 108], as membrane associated enzymes function less efficiently[105, 109].

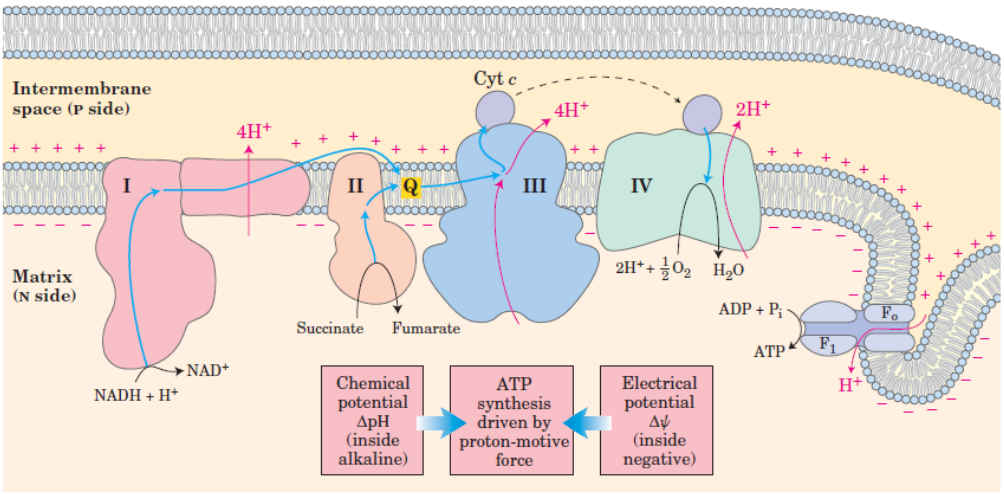

Figure 2: The Electron Transport Chain [105]

1.3.2 Ischemic Reperfusion Injury: The Important Role of Mitochondria in the Kidneys

During an ischemic insult to the kidney, key mitochondria functions are affected. There is a decreased delivery of oxygen, the final oxygen acceptor in the electron transport chain and thus this decreases the protection afforded by the antioxidant system. Slight insults, or short periods of ischemia may increase the leakage of electrons from the blockage of ETC complexes, increasing the electronegativity of ETC complexes, but it may not be substantial enough to drastically alter the chemiosmotic process in the mitochondria. During these brief ischemic insults to the mitochondria, the damaging effects of increased reactive oxygen species can be reduced following reperfusion and reintroduction of oxygen to the system. A longer ischemic insult can result in longer lasting and more profound changes, such as increased membrane permeability[110] and decreased activity of the complexes, resulting in low ATP production[111].

During an ischemic insult, there can also be an increase in resting cytosolic calcium ions, which results in increased concentration of calcium ions in the mitochondria. ATP is also broken down to AMP, adenosine monophosphate and inorganic phosphate. Due to the inhibition of the inhibition of Na^+/K^+ pump, there is an increase in the level of cytosolic Na^+ ions, leading to a rise in cytosolic $Ca^{2+,}$ resulting in changes in membrane permeability[112-115].

Increased mitochondrial inner membrane permeability can have drastic effects on the cell, as it is a signal for apoptosis[116]. During an ischemic insult, there can be the formation of mitochondrial permeability transition pores (MPTP) in the inner mitochondrial membrane, which not only diminish ATP production through proton leakage but they also increase the permeability of small molecules and water, resulting in matrix swelling and rupture of the outer mitochondrial membrane[117]. Cytochrome c, Smac/DIABLO, apoptosis inducing factor (AIF) are released into the cytosol by the ruptured mitochondria, resulting in apoptosis[118].

1.4 Gene Therapy: An Alternative to Treating and Managing AKI

1.4.1 Overview of Gene Therapy

Gene therapy allows for the delivery of exogenous genes into one's cells in order to correct a genetic mutation, be it inherited or acquired. Modifying one's genes allows for altered protein expression patterns, and it is a promising and growing field of medicine that may hold a cure for many diseases. The Human Genome Project has provided a viable map for efforts to identify genes and potentially develop gene therapy as a viable treatment option.

1.4.2 Recent Developments in Gene Therapy

For diseases with a clear target for therapy, for example CFTR gene in cystic fibrosis, gene therapy presents a promising future. There is the vision that one day the disease can be eliminated or prevented when the gene is delivered to the correct target and the problem fixed efficiently while avoiding the side effects of drugs. Gene therapies cannot only treat genetic diseases, but also cancer, viral infections and neurodegenerative diseases. Many clinical trials are focused on cancer treatment, mostly in phase I or II and focused primarily on the safety of genetic treatment[119]. Recently, gene therapy has improved muscle strength in a dog model of a fatal congenital childhood disease caused by the MIM1 gene mutation[120].

There are two critical factors to be considered in a viable gene therapy treatment: efficiency and sustained expression. In the first of its kind, in 1990, a retroviral vector adenosine deaminase (ADA) gene was transferred into the T cells of two children with severe combined immunodeficiency (ADA-SCID). The genes were able to normalize circulating T cells, as well as other cellular and humoral immune responses. However, after two years of gene treatment, the effects only lasted 4 years[121].

There is also the question of a delivery vehicle or the vector. There are two categories: viral and non-viral. Non-viral vectors involve injecting DNA directly, mixing with polylysine or cationic lipids in order to cross the cell membrane, etc. The advantage of non-viral delivery is that it is safer than viral vectors but there is poor efficiency of delivery as well as transient expression of the gene, rendering it relatively ineffective as a treatment option[122].

Viral vectors utilize specialized molecular mechanism refined by evolution to deliver DNA to cells, allowing for persistent expression of the transgene. On the other hand, there are the concerns of toxicity, immune and inflammatory response, gene control, targeting to the correct tissues, the risk of the vector to recover its ability to cause disease and insertional mutagenesis resulting in the potential to create oncogenes[123].

1.4.3 Vital Aspects for Progress in Renal Genetic Treatments

1.4.3.1 Exogenous Transgene Vectors

The delivery of a safe and effective vector is critical to the success of genetic therapy. It is important to develop capable technologies that allow the vectors to target specific cells and achieve sustained gene expression while minimizing adverse effects.

There are five classes of viral vectors divided into two groups, integrating vectors and nonintegrating vectors. Retroviruses and lentiviruses are integrating vectors that allows for genome integration into host DNA, transducing dividing cells. However, genomes of integrating vectors can be silenced over time and thus, using these vectors does not solve the problem of transient expression. Adenoviruses, adeno-associated viruses and herpes simplex-1 viruses are non-integrating viruses, whose genomes are

not integrated into the host genome but instead exists as extrachromosomal episomes and mediate sustained transgene expression in non-dividing cells[124].

With viral vectors, there are the issues of insertional mutagenesis, gene control, targeting and pathogenic side effects. Non-viral vectors, compared to viral methods are safer to use (lower immunogenicity), safer for repeat administration and easier to produce[125].

1.4.3.2 Efficient Renal Gene Delivery Techniques

However, non-viral vectors cannot pass through cellular barriers to access host DNA[126] and in order to be a viable treatment method, there needs to be a delivery method that facilitates the passage of non-viral vectors across the cell membrane, through the nuclear membrane and onto host chromatin. Non-viral vectors involve injecting DNA directly, mixing with polylysine or cationic lipids in order to cross the cell membrane. In an instance of direct injection, naked DNA was injected in in vivo murine with localized, long lasting protein expression effects[127].

Another option is packaging plasmid DNA with liposomes in order to facilitate the delivery of the DNA across membranes and the package is administered via intravenous injection. There is risk of toxicity and immunogenicity. In addition to liposomes, packing with cationic lipids, can deliver the DNA efficiently across the cell membrane as well. Cationic lipids interacts high affinity with cell membranes and once it crosses the membrane, the cationic lipids are dispersed in the cytoplasm, allowing the plasmid to be released from endosomal compartments but the efficiency of DNA transfer into the nucleus has yet to be determined[128]. Another method in use is the use of biocompatible, biodegradable polymer as a plasmid carrier, similar to the mechanism of liposomes and cationic lipids. These methods have been studied in efforts to treat cystic fibrosis and cancer but transfection efficiency is still an issue[129].

There are challenges that need to be overcome in order for efficient and persistent gene transfer and expression in the kidney, due to its unique and complex anatomy. The glomerular filtration barrier blocks the transgene's accessibility to the apical domain of epithelial cells, and that limits the uptake of the transgene[130-132]. Proximal tubule epithelial cells can work as potential transgene target due to their capacity to endocytose exogenous materials. In adenovirus delivery via different methods, the levels of gene expression differed across renal cell types and regions of the kidney, possibly due to the differences in transgene infusion site, volume and rate of delivery, organ temperature and use of vasodilators. The types of deliveries include arterial injections in normal and cystic rats [133], pelvic catheter infusions [134] in normal rats and tail vein [135] and cortical micropuncture injections in uninjured animals[136].

One method of delivery that holds promise, addressing the issue of efficient delivery to the unique structures of the kidney, is hydrodynamic fluid delivery, as it increased vascular permeability to delivery exogenous substances throughout the kidney[137].

1.4.3.3 The Construction of Non-Viral Vectors

There is still the issue of the vehicle for the gene of interest and plasmids, circular double stranded DNA molecules, hold promise. These extrachromosomal DNAs occur naturally in bacteria and yeast. There are three important elements to a plasmid vector: 1) an origin of replication to allow for replication in the bacterial cells (for amplification and subsequent purification), 2) a selectable marker, usually a drug-resistance gene, 3) and a region for DNA of interest insertion. Expression vectors also require specific sequences that allows for protection, insertion and expression:

1) a polyadenylation tail in order to protect mRNAs from exonucleases and ensure translational termination and stabilize mRNA

2) Kozak sequence for ribosome assembly
3) Promotor for initiating transcription for the vector's transgene
4) reporter genes for identifying that the plasmid contains inserted DNA sequence
5) Targeting sequence for targeting the expressed protein to specific locations
6) Protein purification tags for purifying the expressed protein.

One issue is that if the plasmid containing the drug resistant gene is delivered and expressed in a subject, the subject may develop resistance to the specific antibiotic[137].

1.5 Hydrodynamic Fluid Delivery: A Novel Exogenous Gene Delivery Technique

1.5.1 Hydrodynamic Fluid Delivery

In the late 1990s, hydrodynamic fluid delivery was developed to deliver plasmid DNA to whole animals through intravascular injection. The unique characteristic of fluid pressure generated in vessels allowed for enhanced endothelial and parenchymal cell permeability[138-140]. This new delivery approach for non-viral genes seemed efficient, safe and convenient, minimizing the downsides of other delivery methods and viral genes. There have been instances shown when hydrodynamic delivery enabled sustained transgene expression in transfected animals, reaching therapeutic levels[141-148]. Early studies involving other organs like the liver established this method as a potentially effective method for delivery and thus this procedure can be adapted and extrapolated for use in other organs[149-153].

1.5.2 Mechanism of Hydrodynamic Fluid Delivery

The purpose of hydrodynamic fluid delivery is to increase fluid flow from pressurized injections, generating transient pores in the capillary endothelium and epithelium[154]. Theoretically, the increased fluid flow and disruption of physical barriers allows delivery of target DNA to access the plasma membranes of the surrounding parenchyma cells. Then, when the pressure is removed, these exogenous materials are trapped inside the parenchyma cells, as the physical barriers are reformed by the capillary endothelium[155-157].

1.5.3 Characterizations of Various Hydrodynamic Gene Delivery Techniques

In previous work done by Dr. Corridon in the Atkinson lab, hydrodynamic fluid delivery was attempted in several ways in order to optimize a delivery technique that maximizes efficiency of the expression of the transgene while minimizing damage to the kidney tissue. Large molecular weight dye 150kDa FITC was used as a molecular probe to visualize a renal capsule injection into rat kidneys. A significant amount of large molecular weight dye was found in the vasculature but the distribution was uneven and confined to the injection site. Catheter-based renal artery injections were then attempted, where a live rat received a hydrodynamic injection of the large molecular weight molecule, infused through a ligated renal artery via a PE-50 catheter. There was widespread distribution of the dextran molecules, within the lumens of proximal and distal tubules as well as internalized within the proximal tubule epithelial cells. However, there were signs of significant renal injury in the form of constructed vessels, occluded lumens and a large concentration of blebs within the lumens.

Since the renal artery hydrodynamic catheter-based injections resulted in successful and widespread distribution of molecular probes but resulted in significant renal injury, catheter-based renal vein injections were attempted with Texas red-labeled albumin on live rat kidney. There was significant renal injury observed as well. Then,

hydrodynamic fine needle renal arterial injections were attempted with low molecular weight (5kDa FITC dextran) exogenous probes, resulting in an uneven distribution of dextran molecules, which were found endocytosed by proximal tubule epithelial cells in low amounts. Significant injury was observed as well, as there was patchy distribution of dextran, fluorescent debris and non-fluorescent blebs within proximal and distal tubule lumens. When the hydrodynamic arterial injection with single aorta and renal vein clamp was repeated with large molecular probes (150kDa FITC dextran), followed by a subsequent long period of jugular vein infusion of 150kDa TRITC dextran, there was significant amount of blood loss to the injection site. The probes were unevenly distributed within the glomerular and peritubular capillaries, with a more uniform fluorescent signal within the proximal tubule epithelial cells.

The focus was then shifted to hydrodynamic fine needle renal venous injections with small (3kDa Cascade Blue or 4kDa FITC) and large dextrans (150kDa TRITC). Using a 30-gauge needle, these molecular probes were injected at different infusion rats ranging from 10 seconds to 4 minutes with and without vascular clamping. Widespread uptake of both dextrans were observed in vivo, with low molecular dextran molecules bound to brush border membranes of the proximal tubular epithelia. Internalization of the low molecular weight probes were observed in the epithelial cells and concentrated levels of the dextrans appeared within distal tubule lumens. Large molecular weight dextran were widely distributed within the vasculature, internalized by tubular cells and unexpectedly filtered through tubules. Proximal tubule epithelial cells have incorporated low and high molecular weight dextrans along basolateral and apical membranes. After the renal vein hydrodynamic injection, normal renal tissue integrity is restored, as shown by restriction of large TRITC dextran molecules to vascular compartments during a jugular infusion of 150kDa TRITC molecules.

When plasmid eGFP DNA were hydrodynamically injected through the renal vein into rat kidneys without vascular clamps, there were limited levels of transgene

expression within the superficial cortex as well as the renal medulla. When the hydrodynamic injections were repeated with vascular clamps (Figure 6), there was robust expression of GFP and GFP-tubulin in live rat kidneys. Transgene expression was visualized in distal and proximal tubule epithelial cells. The GFP fluorescence intensified along the brush border of cells. Hydrodynamic delivery also facilitates the expression of a variety of plasmid vectors in live rat kidneys including EGFP-occludin, H2B-tdTomato and EGFP actin[137].

Hydrodynamic renal vein injections were also attempted using baculovirus and adenoviruses in addition to plasmids. When the percentage of nephron cross-sections that were expressing the transgene was measured, expression of the transgene was the highest in the superficial renal cortex (78% to 86%) and cortico-medullary junction. The high level of eGFP fluorescent protein persisted in the superficial region of the cortex for up to 4 weeks (28-68%). Serum creatinine in rats remained within normal baseline levels (0.3-0.5mg/dl) throughout the measurement period of up to 14 days after the hydrodynamic fluid delivery, with no significant difference between rats that received saline and rats that received vectors. Thus, transgene expression appears to be relatively long lived and minimized injury using a hydrodynamic fluid retrograde renal vein delivery with cross clamps[137].

1.6 Isocitrate Dehydrogenase 2 (IDH2)

1.6.1 IDH2: An Important Mitochondria Protein

Isocitrate dehydrogenase 2 is a member of isocitrate dehydrogenases (IDHs), a group of proteins with crucial functions and catalyzes the oxidative decarboxylation of isocitrate to alpha-ketoglutarate[158]. There are three IDH genes, IDH1, IDH2 and IDH3. IDH1 and IDH2 are NADPH-dependent enzymes and share similar structure[159]. IDH3 is a NAD+-dependent enzyme located in the mitochondrial matrix and participates in the Krebs cycle. IDH1 and IDH2 catalyze reversible reactions of decarboxylating isocitrate to 2-oxoglutarate while reducing NADP+ to NADPH, while IDH3 irreversibly decarboxylates isocitrate to 2-oxoglutarate while reducing NAD+ to NADH[160].

IDH1 is localized to the cytoplasm and peroxisomes. It is highly expressed in the liver and besides having the ability to catalyze the conversion of isocitrate to alpha-ketoglutarate, IDH1 is also shown to be involved in lipid and glucose metabolism[161, 162] as well as the cellular defense against ROS and radiation [163-165]. IDH2 is found in the mitochondrial matrix and highly expressed in heart, muscle and activated lymphocytes[166]. IDH2 regulates the citric acid cycle and protects cells against oxidative stress[167]. Mutations in IDH1 and IDH2 contribute to tumorigenesis and cancer progression through indirect effects on the overall mutation rate in gliomas and acute myeloid leukemia[168].

1.6.2 Proteomics and the Roles of IDH2 in Protecting Renal Function

Ischemic preconditioning (IP) is a method used to confer resistance to further injury caused by the loss of blood supply and oxygen. When ischemia is induced in a rat kidney for a brief period of time and restored, the kidney is protected from a subsequent ischemic insult for an extended period of time, as measured by serum

creatinine measurements[89]. The mechanism is not yet fully understood but one proposed mechanism is that IP alters the expression of mitochondrial proteins in response to the preconditioning event, allowing for better recovery in subsequent insults. Proteomic analysis carried out by Dr. Bacallao, Dr. Basile and Dr. Witzmann showed that several proteins in the renal mitochondria were elevated beyond baseline levels following a preconditioning event (Table 2). The two most elevated proteins, IDH2 and SULT1C2, were selected for further analysis. Dr. Corridon performed several experiments demonstrating that the hydrodynamic delivery of IDH2 genes can protect the kidney from AKI generated from IRI.

Table 2: Proteomic Screen listing changes in protein expression in rat kidney mitochondria fractions following IRI[137].

Gene	Gene Name	Fold	p-value
SULT1C1	Sulfotransferase 1C2-like	2.4	0.40
IDH2	Isocitrate dehydrogenase (NADP) mitochondrial	2.2	0.31
HIST1H2BC	Histone H2B	2.0	0.25
C1QBP	Complement component 1 Q subcomponent-binding	1.9	0.26
ACOT2	Acyl-coenzyme A thioesterase 2, mitochondrial	1.5	0.53
ACSL1	Long-chain-fatty-acid-COA ligase 1	1.7	0.29
THUMPD2	THUMP domain containing 2 isoform 1	-3.0	0.09
MTCH2	Mitochondrial carrier homolog 2	-1.9	0.27
HSDL2	Hydroxysteroid dehydrogenase-like protein 2	-1.6	0.37
SLC25A13	Solute carrier family 25, member 13	-1.6	0.13
NDUFA5	NADH dehydrogenase (ubiquinone) 1 α subcomplex subunit 5	-1.6	0.33
SUCLG1	Succinate-COA ligase, alpha subunit	-1.6	0.04
ISCA2	Iron-sulfur cluster assembly 2 homolog	-1.3	0.01

In these experiments by Dr. Corridon, serum creatinine measurements were used a standard biomarker for evaluating renal function. Creatinine is a byproduct of normal muscle metabolism and is excreted by the kidney at a relatively constant rate.

When kidney function is impaired, nephron capacity to clear serum creatinine is limited. Less serum creatinine is excreted[29], thereby increasing serum creatinine levels.

Dr. Corridon first performed hydrodynamic retrograde renal injections of either saline or IDH2 plasmid into Sprague-Dawley rat kidneys, and at day 7, they were subjected to a moderate ischemia-reperfusion injury (IRI). Serum creatinine (sCR) measurements were taken for a week after enduring IRI. The sCR measurements showed that the hydrodynamic delivery of IDH2 or SULT1C2 genes was sufficient to ameliorate the effect of an IRI in these rats, mirroring a similar response when the treatment at day one was a preconditioning event. sCR levels remained within normal levels despite the moderate injury as compared to rats that received hydrodynamic or tail injects of saline or fluorescent proteins.

Since IDH2 and SULT1C2 are postulated to have an effect in mitochondria, Dr. Corridon evaluated mitochondrial activity in vivo using two-photon microscopy and mitochondria potential sensitive dye tetramethyl rhodamine methyl ester (TMRM). TMRM is a fluorescent probe which binds to the inner and outer layers of the inner mitochondrial membrane and as an assay, allows us to monitor the membrane potential changes in the mitochondria.

In Sprague Dawley rats, jugular vein infusions of the TMRM dye was used to gain insight into mitochondria function. In normal rats, there was instantaneous and non-uniform basolateral TMRM uptake in the proximal and distal tubule epithelia cells. After subjecting the rats to 30-45 minutes of unilateral ischemia (corresponding to a moderate injury) via a ligature placed around the left renal pedicle of the kidney that received the hydrodynamic injection, there were abrupt decreases in TMRM fluorescent signals. The TMRM signals were eventually reduced to autofluorescent levels about 30 minutes after ischemia and this illustrates the rapid depolarization of mitochondria generated by ischemia.

However, during the restoration of blood flow, there was a gradual increase in the TMRM signal, showing mitochondrial repolarization in vivo. The TMRM signal returned to its initial elevated levels about 15 minutes after reperfusion. The increase in TMRM fluorescence showed the regeneration of mitochondrial potential but it was not uniformly observed throughout the kidney after reperfusion, showing that moderate IRI was significant enough to impair some mitochondrial repolarization function. This results compare to previous reported results[169].

In comparison to rats which have previous received a hydrodynamic retrograde renal injection of either IDH2 or SULT1C2 plasmid vectors, as those that have received IP, the TMRM fluorescent signal intensities were significantly greater than those measure in sham and vehicle rats (injected with saline). This shows that there is potential increase in mitochondrial activity related to the upregulation of the mitochondrial enzymes facilitated by the hydrodynamically injected IDH2 and SULT1C2 genes as well as IP. The upregulation in protein expression of IDH2 and SULT1C was confirmed using Western analysis and supports the previously described TMRM data.

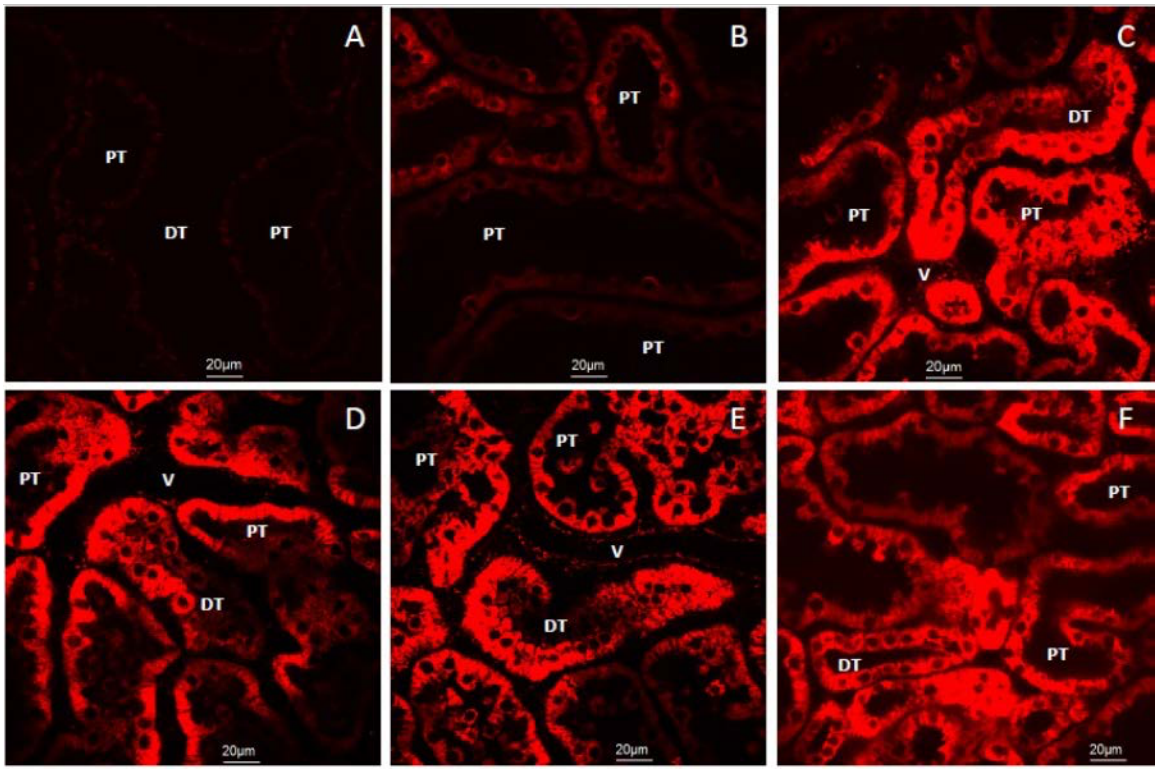

Figure 3: Mitochondria Membrane Potential activity examined in nephron segments of live rats visualized with TMRM dye [137]

(A) – baseline autofluorescence in a normal rat that has not received the TMRM dye. (B) Saline injected. (C) IDH2 plasmid injected. (D)SULT1C2 plasmid injected. (E) SULT1C2 plasmid injected in unilaterally nephrectomized (right kidney removed) rats. (F) was subjected to ischemic preconditioning and did not receive any injections. TMRM were introduced via the jugular vein after the kidneys were subjected to 30mins of ischemia, 7 days after receiving hydrodynamic fluid delivery or IRI treatment. The kidneys were imaged about 15-20minutes after the jugular vein infusion of TMRM and renal blood flow restored (taken from Dr. Peter Corridon's thesis[137]).

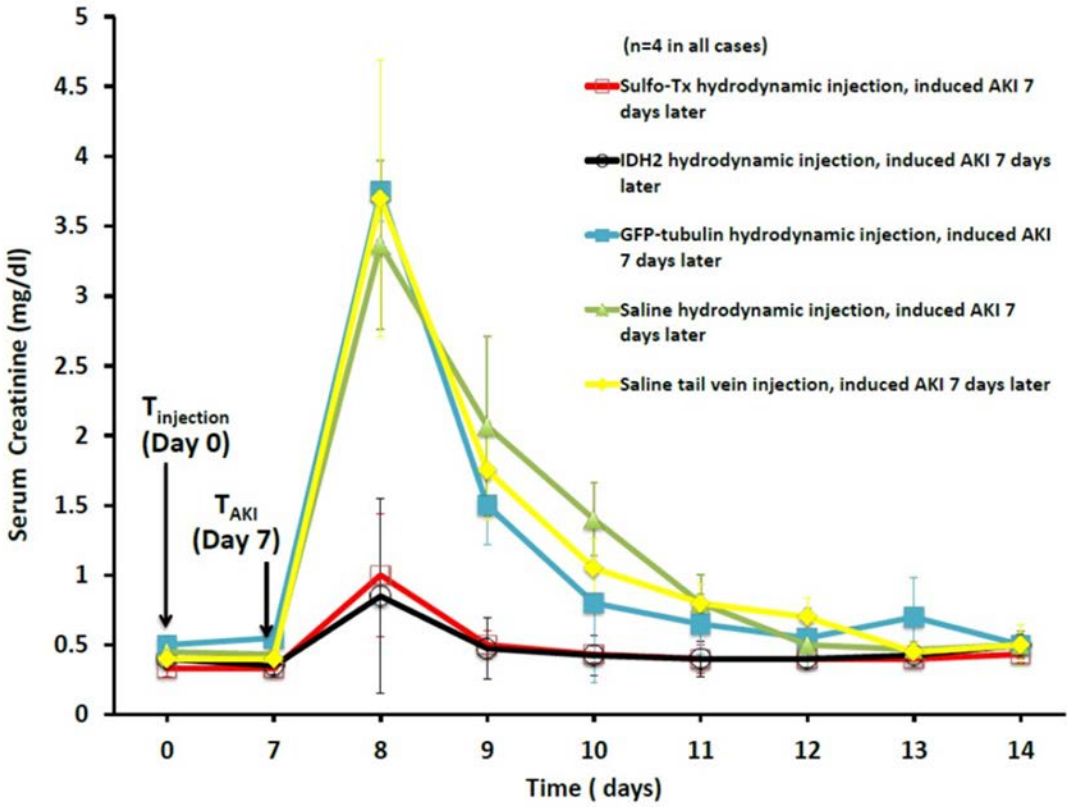

Figure 4: Time Progression of Serum Creatinine (sCR) values in hydrodynamically injected rats.[137]

At day 0, rats are hydrodynamically injected with various plasmid solutions or saline as denoted by the legend. At day 7, rats are injured via moderate IRI and sCR values are taken every day post injury up to seven days. Rats injected with IDH2 and SULT1C2 enzymes have lower sCR values one day post-IRI, thus showing that the enzymes protect the kidney from moderate forms of AKI. (Taken from Dr. Peter Corridon's thesis[137])

1.7 In Vitro Assays for Mitochondrial Respiration

In 1955, Chance and Williams defined respiratory control states and designed a protocol for mitochondrial activity measure with isolated mitochondria. The in vitro measurement of mitochondrial oxygen consumption uses various manipulations in order to monitor several different functional states (figure 5). The first state is state 1, where mitochondria are added to an isosmotic medium containing phosphate. Respiration is slow due to a lack of substrate in the medium. Substrates like succinate,

pyruvate, isocitrate, alpha-ketoglutarate and malate are then added for state 2. Mitochondrial oxygen respiration in state 2 is still low in the presence of substrate due to the lack of ADP, as state 2 represents the oxygen consumption before the addition of ADP. In state 3, oxygen consumption is stimulated by the addition of ADP and respiration is activated with the presence of both the substrate and ADP. Respiration is ramped up and oxygen consumption can be represented as a function of slope. ADP is used up by the phosphorylation of ADP to ATP by the mitochondria. Respiration slowly decreases at the end of state 3 due to the limiting factor of ADP. In state, 4, ADP is a limiting factor and respiration declines. In state 5, there is a lack of oxygen in the closed oxygen monitor chamber and that restricts respiration[170]. Mitochondrial oxygen respiration is measured as a consumption rate of oxygen during state 3 with the presence of both substrate and ADP [170].

The respiratory control ratio (RCI) can also be calculated by taking the ratio of state 3 to state 4. The RCI is a quantification of mitochondria coupling and the higher the ratio, the tighter the coupling between the mitochondrial respiratory chain and oxidative phosphorylation. The ADP:O ratio can also be calculated, and it is a ratio between the amount of ADP phosphorylated and the amount of oxygen consumed during state 3[170].

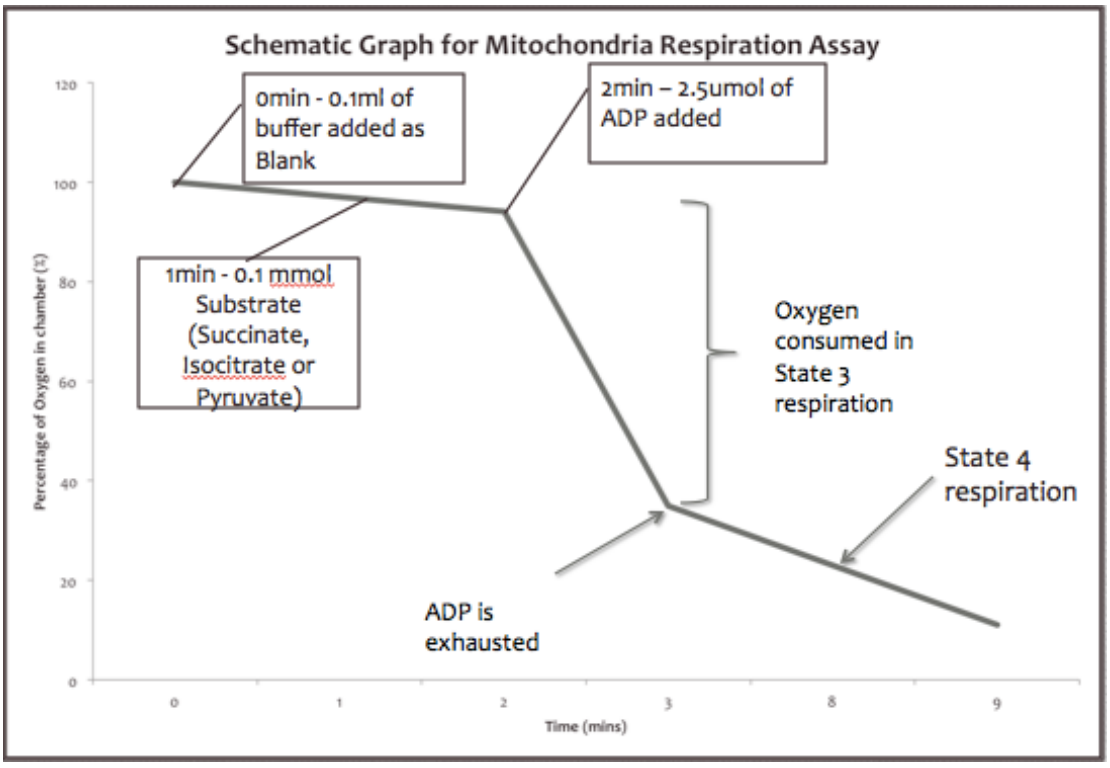

Figure 5: Schematic graph of oxygen consumption assay.

At t=0min, 0.1ml blank (terzic buffer) is added to the chamber. At t=1min, a substrate (0.1mmol) is added (succinate, isocitrate or pyruvate). At t=2mins, 2.5umol of ADP is added. State 3 is the active respiration state, when mitochondria are actively converting ADP to ATP. State 4 occurs when ADP is exhausted and respiration slows down.

1.8 Hypothesis

Our hypothesis is that mitochondria in preconditioned rats, or rats that have received a prior ischemic injury two weeks before ischemic-reperfusion injury before sacrifice, can mitigate the second injury better than rats that have not received the preconditioning injury event. This alteration in mitochondria oxygen consumption is also seen in mitochondria from kidneys injected with hydrodynamically injected IDH2 plasmids.

In a previous study conducted by our collaborators, preconditioned rats were shown to be resistant to moderate ischemic injury as shown by lower serum creatinine values as compared to rats that have not been treated with a preconditioning event of ischemic injury. In these preconditioned rats, IDH2 has been shown to be upregulated in the kidneys of rats that are resistant to moderate ischemic injuries, according to western blot analysis. Since the IDH2 enzyme plays a critical role in the citric acid cycle, we hypothesized that the mitochondria could be the key factor in facilitating such a resistance to further insults, and thus in a two-pronged effort, we hypothesize that 1) mitochondria oxygen consumption in preconditioned rat renal mitochondria will be equal or better as compared to controls, and better compared to renal mitochondria isolated from injured rat kidneys 2) mitochondria oxygen consumption in preconditioned rat renal mitochondria will perform similar to IDH2 hydrodynamically injected kidneys, and better than injured, saline hydrodynamically injected kidneys.

CHAPTER 2. MATERIALS AND METHODS

2.1 Plasmid Vectors

Plasmid DNA was isolated using the Qiagen Maxi Prep systems (Qiagen, Chatsworth, CA). The plasmids were encoded with enhanced green fluorescent protein eGFP-actin (Clontech Laboratories, Mountain View, CA), non-fluorescently labeled plasmid vectors that encode mitochondrial enzymes IDH2 and sulfotransferase (OriGene Technologies, Inc, Rockville, MD). The IDH2 vector was an ORF clone of Homo sapiens isocitrate dehydrogenase 2, a nuclear gene encoding mitochondrial protein as transfection-ready DNA. The sulfotransferase vector was an ORF clone of Homo sapiens galactose-3-O-sulfotransferase 2 (GAL3ST2) as transfection-ready DNA. For these hydrodynamic injections, for hydrodynamic injections, the dose was 1 μg of plasmid DNA/g of body weight diluted to a total volume of 0.5ml with saline.

2.2 Mouse Kidney Cell Culture

S3 segment of the proximal tubule epithelial cells were cultured in a mixture of 500ml of essential media with 7.5% sodium bicarbonate, 7% fetal bovine serum and 1% of streptomycin (Fisher Scientific, Pittsburgh, PA). The cells were grown in a 37°C, 5% CO_2-38% CO_2 humid incubator.

2.3 Cell Culture Transfection

Immortalized cell cultures were grown in 35mm glass bottom, no.1.5 coverslip dishes, with standard thickness of 0.17 millimeters (corning Inc., Corning, NY). We used the Effectene Transfection Reagent protocol provided by Qiagen (Valencia, CA), for plasmid-based transfections. We transfected cells at a multiplicity of infection (MOI) of 10:1, and a 24-hour incubation period using both types of viral vectors.

2.4 DNA Agarose Gel Electrophoresis

The E-Gel® with SYBR Safe 1.2% agarose gel (Invitrogen, G521801) was inserted into the E-Gel® PowerBaseTMv.4. Each well of the gel was loaded with 500ng plasmid DNA. The electrophoresis time was set as 30 min.

2.5 Live Sprague Dawley Rats

Male Sprague Dawley rats (Harlan Laboratories, Indianapolis, IN) with weights ranging from 250g to 350g were used for these studies. The rats were given free access to standard rat chow and water throughout the studies. Experiments were conducted in accordance with National Institutes of Health Guidelines and were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee

2.6 Transmission Electron Microscopy

The specimens were fixed in 2%Paraformaldehyde/2%Glutaraldehyde in 0.1M phosphate buffer. After fixation the specimens are rinsed with phosphate buffered saline (PBS) followed by post fixation with 1% osmium tetroxide in phosphate buffer for one hour. After rinsing again with PBS the tissue specimens are dehydrated through a series of graded ethyl alcohols from 70 to 100%. After dehydration the specimens were infiltrated with 2 changes of 100% acetone and a 50:50 mixture of acetone and the

embedding resin (Embed 812, Electron Microscopy Sciences, Hatfield, PA) for overnight. The next day the specimens are transferred to fresh 100% embedding media for a minimum of 2 hours, then embedded in a fresh change of 100% embedding media. Following polymerization overnight at 60°C the blocks are then ready to section. Thin sections were cut (70-80nm), stained with uranyl acetate and lead citrate, then viewed on a Tecnai BioTwin (FEI, Hillsboro, OR) with digital images taken with an AMT (Advanced Microscope Techniques, Danvers, MA) CCD camera.

2.7 Ischemia/Reperfusion Injury

2.7.1 Bilateral Clamp Model

Rats were anesthetized with intraperitoneal injections of 50mg/kg pentobarbital (Hospira, Inc., Lake Forest, IL and CustomMed Apothecary, Indianapolis, IN) or ketamine xylazine ace cocktail. They were then placed on a heating pad to maintain normal physiological temperature. Using a standard model to general renal ischemia-reperfusion injury, bilateral renal pedicle clamps (Fine Science Tools, Foster City, CA) were applied to cut off blood flow for a period of 40 minutes. These clamp periods correspond to moderate kidney injury[154, 171]. After 40 minutes, the clamps were removed to restore renal blood flow and the animals were allowed to fully recover. 8 hours post surgery, the rats were injected subcutaneously with buponorphine (0.05mg/kg) to manage pain.

2.7.2 Unilateral Clamp Model

Rats were anesthetized with intraperitoneal injections of 50mg/kg pentobarbital (Hospira, Inc, Lake Forest, IL and Custom Med Apothecary, Indianapolis, IN) or ketamine xylazine ace cocktail and placed on a heating pad to maintain normal physiological

temperature. Using a standard model to general renal ischemia-reperfusion injury, the left renal pedicle were clamped with a microserrefine clamp (Fine Science Tools, Foster City, CA) to occlude blood flow for a period of 40 minutes, which corresponds to moderate acute kidney injury. At the end of each injury period, the clamp was removed to reinstate renal blood flow according to reperfusion model.

2.7.3 Ischemic Preconditioning

Rats were again anesthetized in the manner described above and subjected to unilateral pedicle clamps to occlude renal blood flow for a period of 40 minute. The incisions were temporarily closed during ischemia. After 40 minutes, the clamp was removed to reinstate renal blood flow for 1 hour before the kidneys were removed and the rats sacrificed.

2.8 Hydrodynamic Retrograde Renal Vein Delivery

2.8.1 Retrograde Renal Fine-Needle Injection with Vascular Clamps

Rats were anesthetized by inhaled isoflurane (5% in oxygen, Webster Veterinary Supply, Devens, MA) and then placed on a heating pad to maintain normal physiological temperature. Once the animal was fully sedated, the abdominal region was shaved and sanitized with Betadine Surgical Scrub (Purdue Products L.P., Stamford, CT) solution. It was then restrained on a heating pad. A midline incision was made to expose the left kidney. The renal artery and vein were separated and the renal artery was clamped first before the renal vein was clamped using micro-serrefines. The vein was isolated with a 3-0 silk loop and elevated. We performed a retrograde infusion of either tissue dye or transgenes at an approximate rate of 0.1ml/s into the renal vein, using a 30-gauge needle. We then removed the needle and applied pressure to the injection site, with a

cotton swab to induce hemostasis. The clamps were then removed (venous before arterial clamp) to restore renal blood flow. The total clamping period lasted 3 minutes. The midline incision was closed and the animal was allowed to fully recover. 8 hours post surgery; the rats were injected with buponorphine.

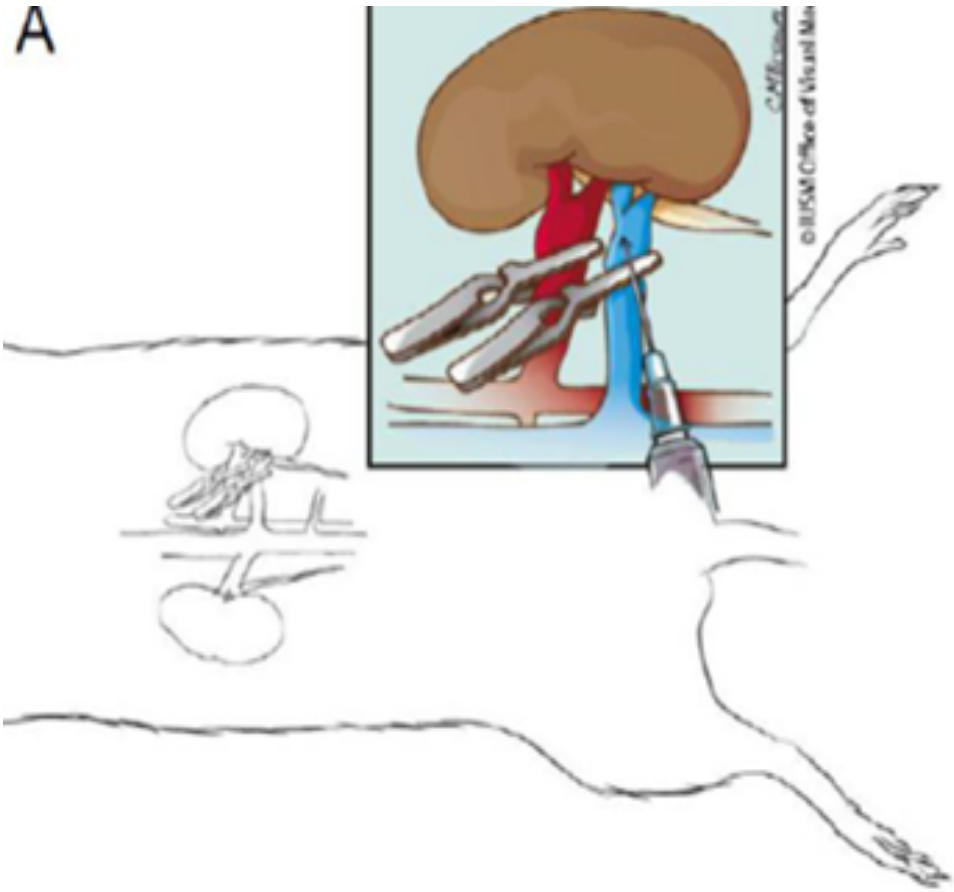

Figure 6: Schematic of hydrodynamic fluid delivery with vascular cross clamps[137].

2.8.2 Parameter for Successful Retrograde Renal Vein Hydrodynamic Injection: Pressure Profile

To characterize the hydrodynamic delivery process and measure pressure changes within the renal vein, we monitored time-dependent pressure profiles during the hydrodynamic injections. A modified double 30-gauge needle was used to connect the

needle to a fluid-filled pressure transducer and data was acquired in real time using data-acquisition software (Biopac Systems, Inc., Goleta, CA).

2.9 Western Blot Analysis

Whole kidneys were extracted from anesthetized rats, which were then euthanized. The cortex from the kidney was excised and homogenized in RIPA buffer (150mM NaCl, 50mM Tris-Cl pH 8.0, 1% Triton X-100, 0.5% Sodium Deoxycholate, 0.1% SDS). The samples were then sonicated and centrifuged. The extracts (3-15µg/lane) were separated through the 10% Tris-Glycine Mini Gels, along with 4µl molecular weight markers (Precision Plus Protein TM Dual Color Standards, BIO-RAD, Cat. #616-0374). Proteins were electroblotted to nitrocellulose membranes. The membrane was blocked by incubation in TBS with 3% FBS for 1hr at room temperature. Membranes were incubated with primary antibody in TBS with 0.3% FBS for 1hour at room temperature. After three washes at 5mins each, the membrane was incubated with appropriate IRDye fluorescent secondary antibody (LI-COR, Lincoln, NE) in antibody dilution buffer at room temperature for 1 hour. After three washes each 5mins, the blots were dried overnight in a black box. The next day, the blots were imaged using the LiCor system.

2.10 Mitochondria Oxygen Respiration Assay

2.10.1 Extracting the Kidneys

The rats were anesthetized with either pentobarbital or ketamine xylazine ace cocktail. A midline incision on the abdomen is made and the appropriate injury performed on the left or right kidney. At the end, the rat is euthanized.

2.10.2 Homogenization

After the kidney was harvested from the rat, the capsule layer was peeled and the cortex was shaved off. At 4°C, the cortex was gently homogenized at 370 rpm with a tight fitting homogenizer in 1ml mitochondrial lysis buffer (250mM sucrose, 20mM HEPES, 10mM KCl, 1.5 mM Mg_2Cl, 1.0mM EDTA, pH 7.9) using a Wheaton Teflon pestle and 15mL homogenization tube.

2.10.3 Mitochondria Purification

The homogenate was spun at 800g for 10mins at 4°C. The supernatant was transferred to another microcentrifuge tube and spun at 10000xg for 10mins 4°C. The supernatant was discarded and the pellet was resuspended in 1mL of Lysis buffer. The process was repeated for 3 washes with the Lysis buffer and kept on ice with caps open at the end of the purification. The concentration of mitochondria fraction was determined by Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Tek Instruments, Inc.)

2.10.4 Mitochondria Oxygen Concentration Measurement

Before commencing each set of mitochondrial fractions, the oxygen electrode of the Oxygen Meter (Model 5300, Yellow Springs Instrument Co.) was calibrated and the membrane changed. The chambers for each sample were surrounded by a temperature-controlled water-jacketed glass chamber maintaining the temperature at 37°C.

The mitochondrial fraction for each kidney was divided into 3 portions (roughly 350µL) and each portion was added to the oxygen chamber with 2.2mL of terzic Buffer (110mM KCl, 5mM K2HPO4·$3H_2O$, 10mM MOPS, 10mM Mg·Acetate, 1mM EDTA, 1µM tetrasodium pyrophosphate, 0.1% BSA, pH 7.15) for a total volume of 2.5mL.

The electrode was gently inserted into the chamber, making sure that there was no air bubbles visible in the chamber. The stir bar was turned on and the oxygen percentage was recorded when the 0.1 ml of the following substances was added every 1-minute in the following order: Terzic buffer (as blank), substrate (1M succinate or 1M isocitrate or 1M pyruvate), and ADP (25mM). The substances were injected by 23-gauge syringed attached to PE-50 tubing to give a final concentration of 10mM in the chamber. At 10minutes, the recording of the mitochondria oxygen respiration was stopped.

2.11 Statistical Analysis

We computed the mean and S.E. for all data sets, and evaluated the statistical significance of our results using the Student's t-test. All statistical analyses were evaluated using 95% confidence threshold.

CHAPTER 3. RESULTS

3.1 Ischemic Preconditioning Protects Sprague Dawley Rat Kidney Mitochondria Against Dysfunction Following Moderate Ischemic/Reperfusion Injury

We first investigated the effect of ischemic preconditioning on respiration rates in mitochondria isolated from the kidney cortex. For the preconditioning part of the study, there are in total of six groups of kidney cortex samples (Table 2). Preconditioning was induced by clamping the pedicle of both kidneys for 40 minutes. Subsequently, animals were subjected to bilateral or unilateral ischemia using the same procedure. Sham surgery was used as a control. Sham Sham (SS) rats underwent sham surgery at day 0 and at day 14, both right and left kidneys are accorded the same treatment and manipulation. In the PC Uni group, rats underwent bilateral ischemic reperfusion injury (IRI) at day 0, and unilateral left kidney IRI at day 14 before sacrifice. In the PC Bi group, rats underwent bilateral ischemic reperfusion injury (IRI) at day 0, and bilateral kidney IRI at day 14 before sacrifice. In the PC S group, the kidneys are the right kidneys of the PC Uni group, which underwent bilateral ischemic reperfusion injury (IRI) at day 0 and sham injury at day 14 before sacrifice. In the SI Uni group, rats underwent sham surgery at day 0 and unilateral left IRI at day 14. In the SI Bi group, rats underwent sham surgery at day 0 and bilateral IRI at day 14. All IRIs are 40 minutes ischemia followed by a 1 hour reperfusion unless stated otherwise. The vehicle without Injury, vehicle with injury and IDH2 groups are data from a previous thesis by Shijun Zhang [172]. Mitochondrial fractions were isolated from the cortices of each kidney and mitochondria are purified from these cortices to be used in the mitochondrial oxygen consumption assay.

Table 3: Summary of the different preconditioning study rat groups.

Group	Group Abbreviation	Surgery at Day 0	Surgery at Day 14	n
Sham Sham #	SS	Sham	Sham	27
Preconditioned, Unilaterally Injured	PC Uni	40min bilateral renal pedicle clamps	40 min unilateral left renal pedicle clamp, 1hr reperfusion	12
Preconditioned, Bilaterally injured	PC Bi	40min bilateral renal pedicle clamps	40 min bilateral renal pedicle clamps, 1 hr. reperfusion	10
Preconditioned without 2nd injury*	PC S	40min bilateral renal pedicle clamps	Sham	12
Sham Injury (Unilateral)	SI Uni	Sham	40 min unilateral left renal pedicle clamp, 1hr reperfusion	9
Sham Injury (Bilateral	SI Bi	Sham	40 min bilateral renal pedicle clamps, 1 hr. reperfusion	8

- Data includes both left and right kidneys. * - Data is derived from right kidney of PC Uni rats, right kidneys are not injured at day 14.

Table 4: Summary of the different hydrodynamic injection study rat groups.

Group	Group Abbreviation	Surgery at Day 0	Surgery at Day 7	n
Vehicle without 2nd injury	VwoI	0.5ml of Saline, hydrodynamically injected	Sham	9
Vehicle with Injury	VI	0.5ml of Saline, hydrodynamically injected	40mins unilateral left renal ischemia	9
IDH2 injection	IDH2	0.5ml of IDH2 plasmid in saline, hydrodynamically injected	40mins unilateral left renal ischemia	9

We measured serum creatinine values following preconditioning or sham surgery, to determine that the desired degree of injury had been achieved, with a rise in serum creatinine indicative of moderate AKI. The serum creatinine values for each of the Preconditioning study groups are checked at day 1, 2, 7 and 14. Serum creatinine is not taken for hydrodynamic injection study groups. For all the preconditioning study groups, serum creatinine levels are elevated and the averages for each time point are shown in figure 6 and 7.

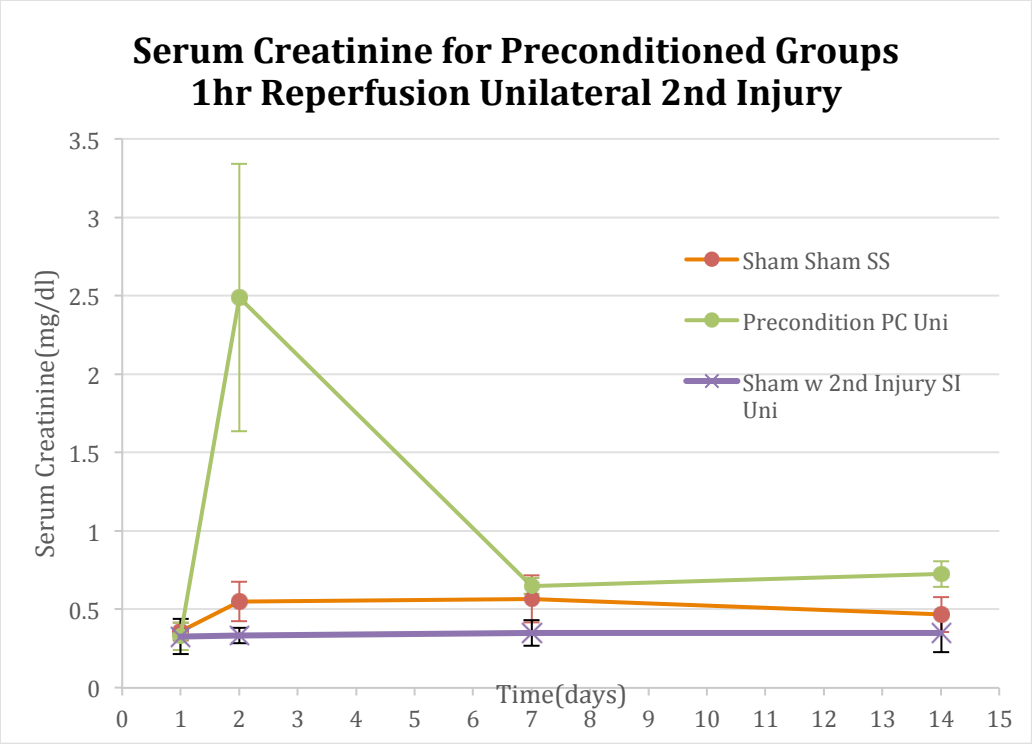

Figure 7: Serum creatinine values of SS, PC Uni and SI Uni groups over 14 days.

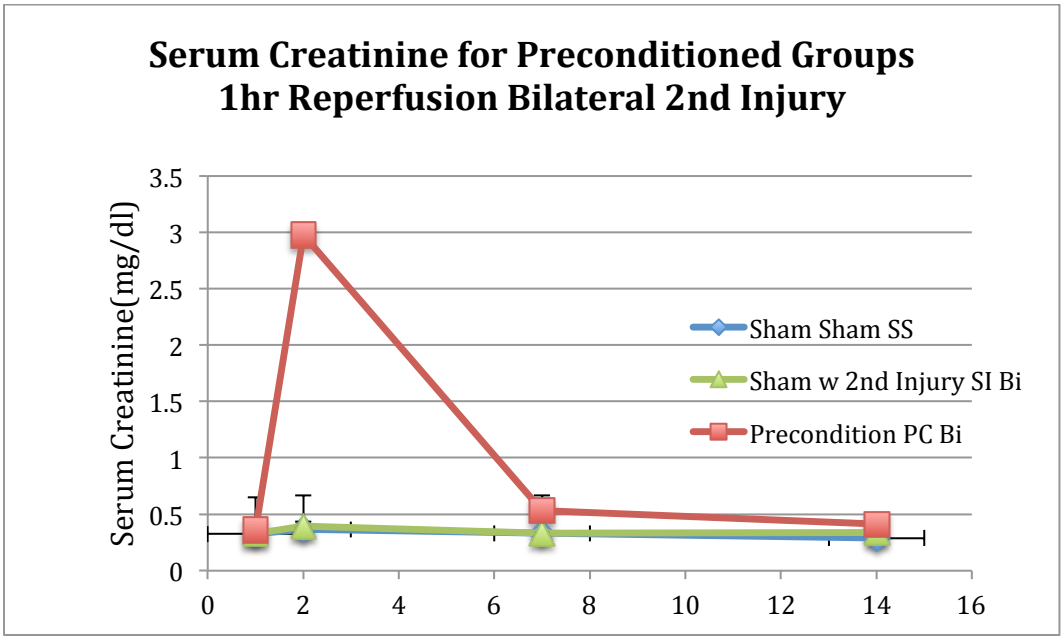

Figure 8: Serum creatinine values of SS, PC Bi and SI Bi groups over 14 days.

Mitochondrial respiration was assayed using three different substrates that enter the Krebs cycle at different points. Using these substrates, the mitochondria oxygen consumption is measured at State III in the respiration cycle. The respiration rates of all groups are shown in Figure 8, and broken down in analysis subsequently. A respiration control index (ratio of State III to State IV) is unable to be achieved by the equipment used in this set of studies, but can be calculated with a more refined protocol and more advanced machines, namely the Oroboros O2k machine (Bioblast, Innsbruck, Austria) or Seahorse XF Analyzer (Seahorse Bioscience, North Billerica, MA). The results of the mitochondria respiration assay can be broken down into various comparisons among the above-described groups of kidneys.

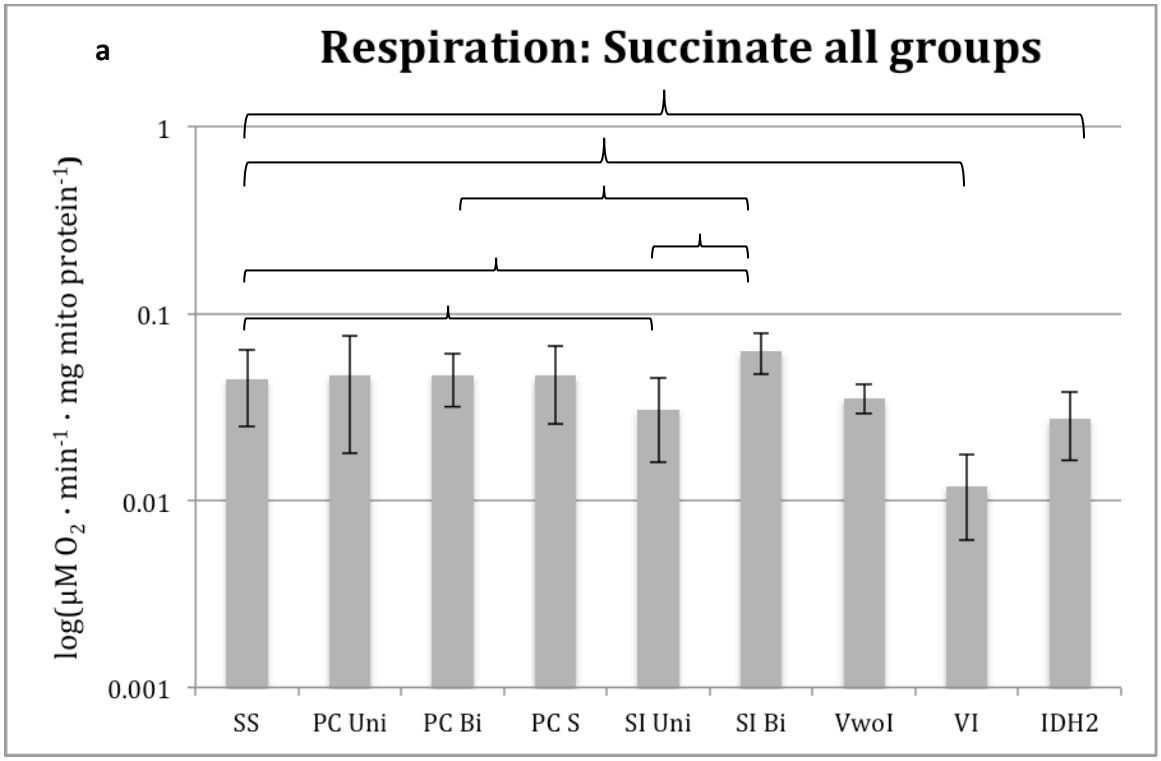

Figure 9: Summary of all respiration rates of all groups of rat renal cortex mitochondria fractions on a logarithmic scale.

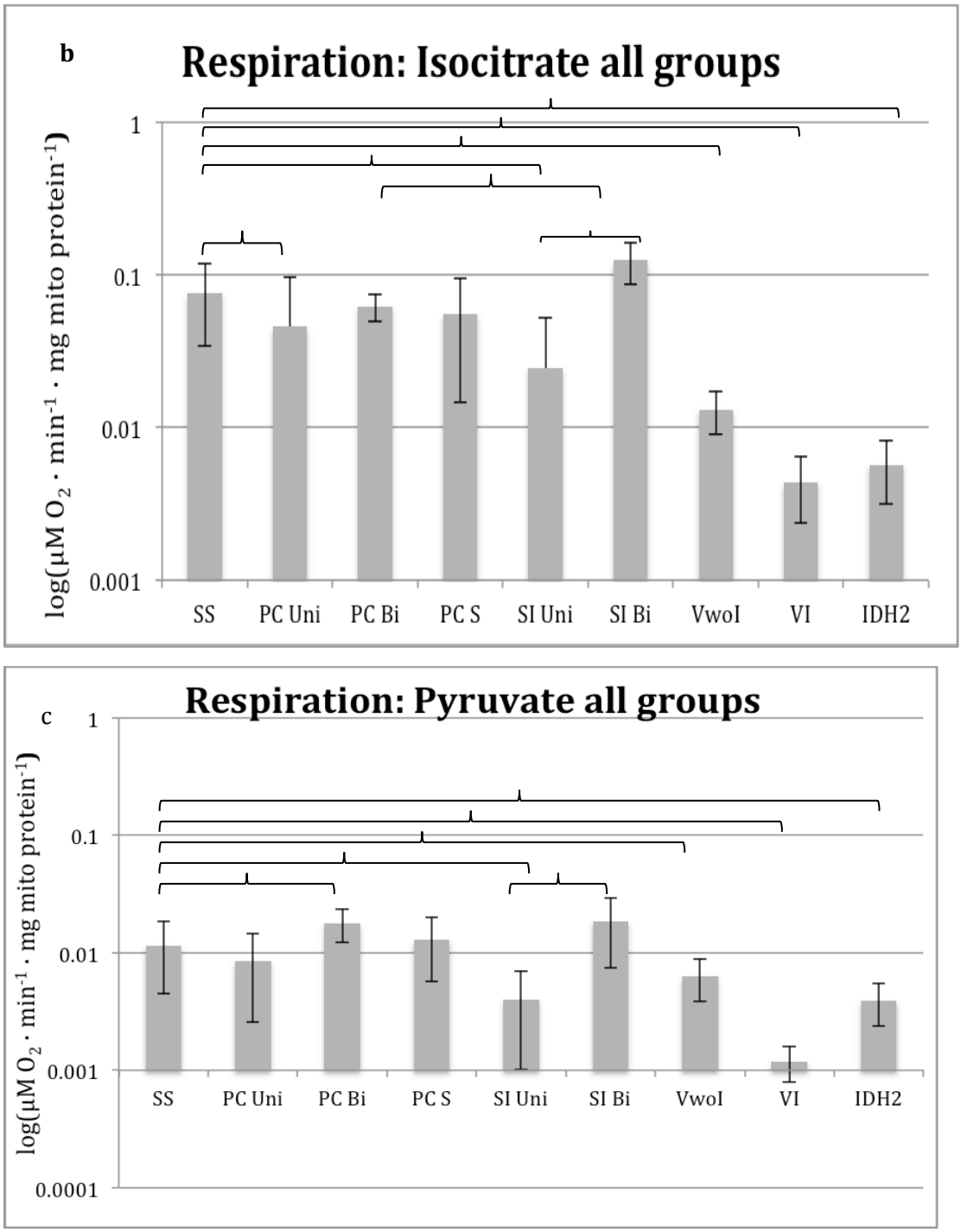

Figure 9: Summary of all respiration rates of all groups of rat renal cortex mitochondria fractions on a logarithmic scale (continued).

Figure 9: Graph 8a corresponds respiration rates of renal mitochondria with the substrate succinate added at t=1min, Graph 8b corresponds to the substrate isocitrate added at t=1min and graph 8c corresponds to the substrate pyruvate added at t=1min. Oxygen consumption is measured in micromole oxygen consumed per minute, per milligram of mitochondria protein (as determined from the Bradford Assay). Refer to table 2 and 3 for legend and n. All brackets over pairs of data values denote a significant difference between the two groups ($p<0.05$)

3.1.1 Sham unilateral injury (SI Uni) rat renal mitochondria respiration rates are depressed as compared to Sham Sham renal mitochondria respiration rates for all substrates.

For all substrates, SI Uni mitochondria respiration rates are significantly different from SI Bi mitochondria respiration rates ($p<0.05$). SI Bi mitochondria respiration rate is significantly elevated above that of sham group SS ($p<0.05$) and SI Uni mitochondria respiration rate is significantly depressed compared to that of sham group ($p<0.05$).

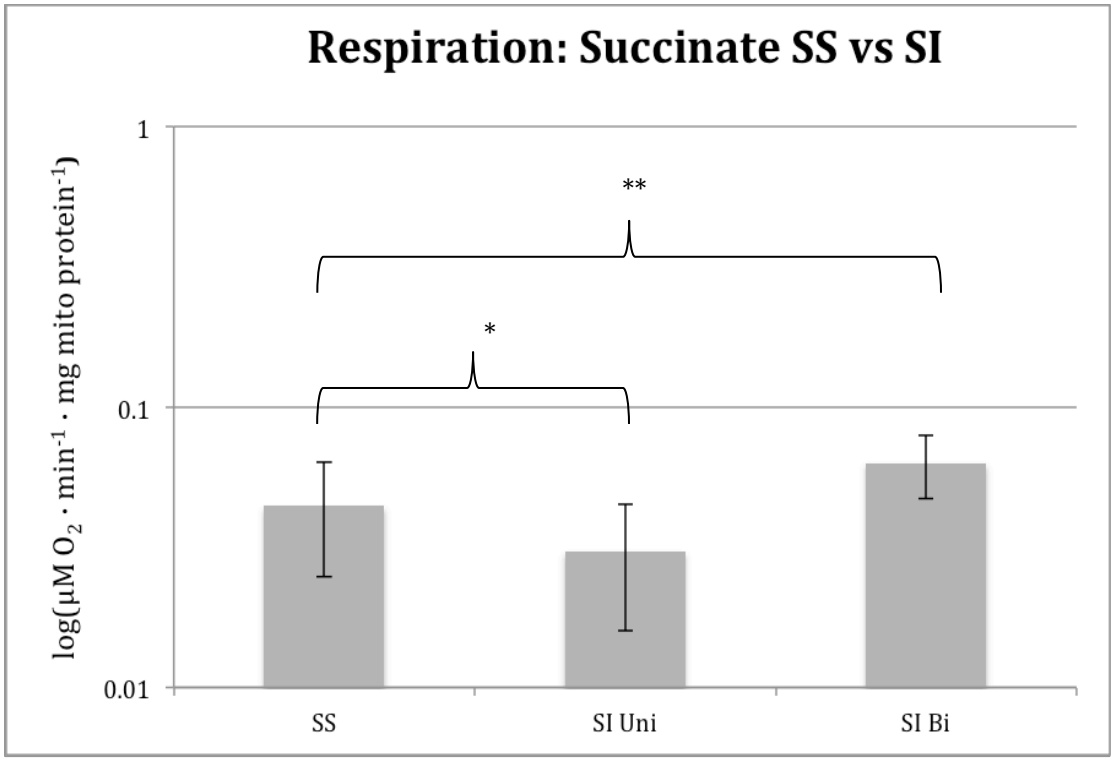

Figure 10: Comparison of respiration rates of Sham Sham (SS) group compared to the two sham injury (SI) groups for the substrate Succinate on a logarithmic scale.

Sham, Unilaterally injured rat kidney cortex mitochondria fractions (SI Uni) and Sham, Bilaterally injured rat kidney cortex mitochondria fractions (SI Bi). *, ** - The SI Uni and SI Bi group are both significantly different from the SS group ($p<0.05$). Oxygen consumption is measured in micromole oxygen consumed per minute, per milligram of mitochondria protein (as determined from the Bradford Assay). SS n=27, SI Uni n=9, SI Bi b=8.

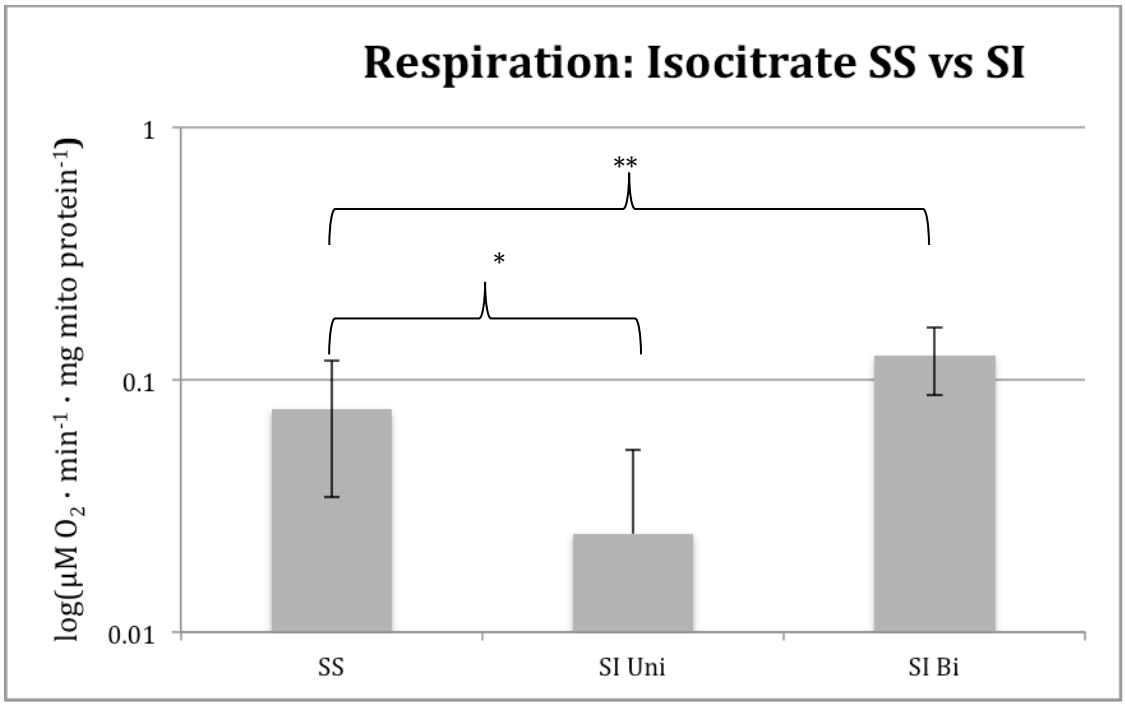

Figure 11: Comparison of respiration rates of Sham Sham (SS) group compared to the two sham injury (SI) groups for the substrate Isocitrate on a logarithmic scale.

Sham, Unilaterally injured rat kidney cortex mitochondria fractions (SI Uni) and Sham, Bilaterally injured rat kidney cortex mitochondria fractions (SI Bi). The SI Uni and SI Bi group are both significantly different from the SS group (*, ** $p<0.05$). Oxygen consumption is measured in micromole oxygen consumed per minute, per milligram of mitochondria protein (as determined from the Bradford Assay). SS n=27, SI Uni n=9, SI Bi b=8.

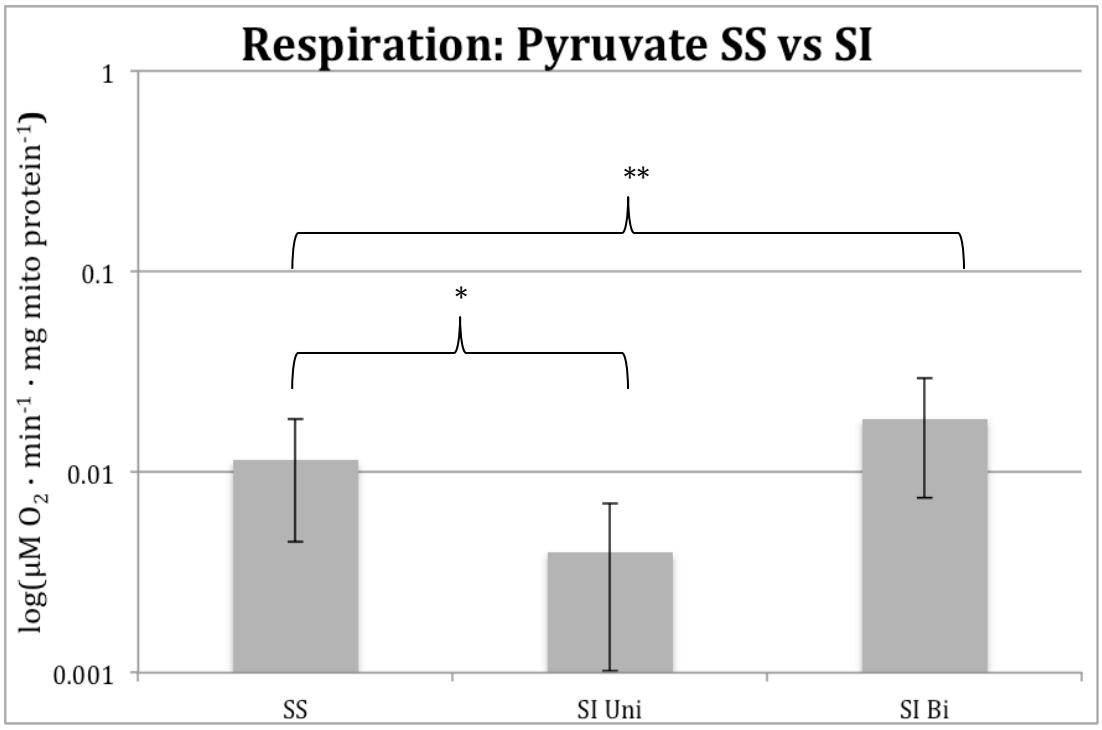

Figure 12: Comparison of respiration rates of Sham Sham (SS) group compared to the two sham injury (SI) for the substrate Pyruvate on a logarithmic scale.

Sham, Unilaterally injured rat kidney cortex mitochondria fractions (SI Uni) and Sham, Bilaterally injured rat kidney cortex mitochondria fractions (SI Bi). The SI Uni and SI Bi group are both significantly different from the SS group (*,** $p<0.05$). Oxygen consumption is measured in micromole oxygen consumed per minute, per milligram of mitochondria protein (as determined from the Bradford Assay). SS n=27, SI Uni n=9, SI Bi b=8.

3.1.2 Preconditioned Unilaterally and Bilaterally Injured Rat Renal Mitochondria respire similar or as compared to sham sham rat renal mitochondria for two substrates: succinate and pyruvate.

Using succinate as a substrate, after ischemic reperfusion injury (IRI), mitochondria respiration of preconditioned unilaterally injured rat renal mitochondria (PC Uni) and preconditioned bilaterally injured rat renal mitochondria (PC Bi) are performing on a similar level to sham sham (SS) renal mitochondria ($p>0.05$). The PC Uni and PC Bi mitochondria has endured not one but two separate events of moderate ischemic injury and yet, the mitochondria respiration for these PC groups compared to sham group SS is indistinguishable.

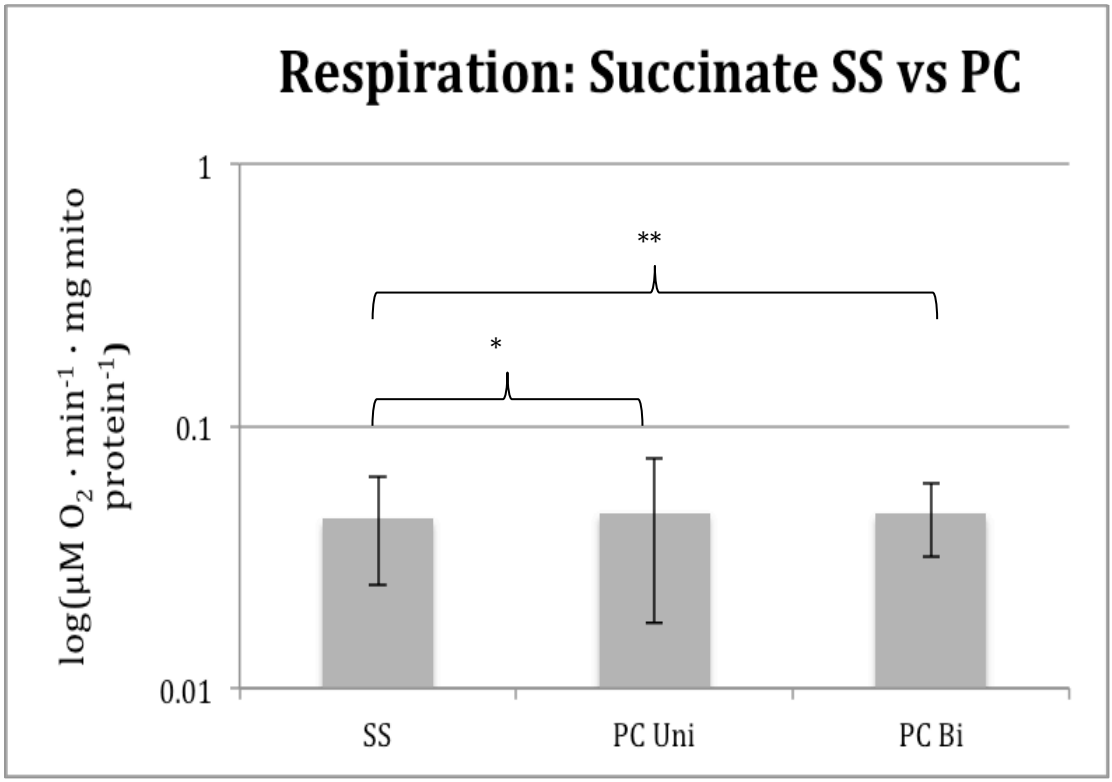

Figure 13: Comparison of respiration rates of Sham Sham (SS) group compared to the two preconditioned groups for the substrate succinate on a logarithmic scale.

Preconditioned, Unilaterally injured rat kidney cortex mitochondria fractions (PC Uni) and Preconditioned, Bilaterally injured rat kidney cortex mitochondria fractions (PC Bi). *, ** - There are no differences between the SS group and each PC group ($p>0.05$). Oxygen consumption is measured in micromole oxygen consumed per minute, per milligram of mitochondria protein (as determined from the Bradford Assay). SS n=27, PC Uni n=12, PC Bi n=10.

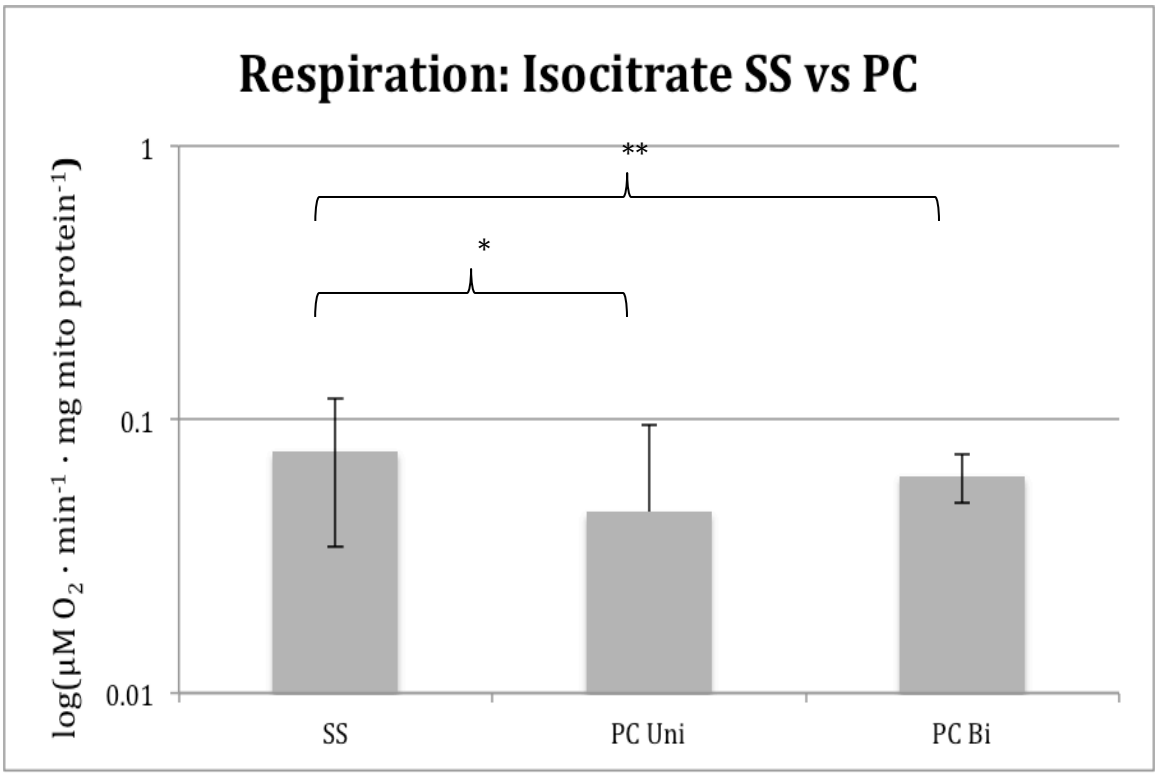

Figure 14: Comparison of respiration rates of Sham Sham (SS) group compared to the two preconditioned group for the substrate Isocitrate on a logarithmic scale.

Preconditioned, Unilaterally injured rat kidney cortex mitochondria fractions (PC Uni) and Preconditioned, Bilaterally injured rat kidney cortex mitochondria fractions (PC Bi). There is a significant difference between the SS group and PC Uni group (*, $p<0.05$). There is no difference between the SS group and PC Bi group (**, $p>0.05$). Oxygen consumption is measured in micromole oxygen consumed per minute, per milligram of mitochondria protein (as determined from the Bradford Assay). SS n=27, PC Uni n=12, PC Bi n=10.

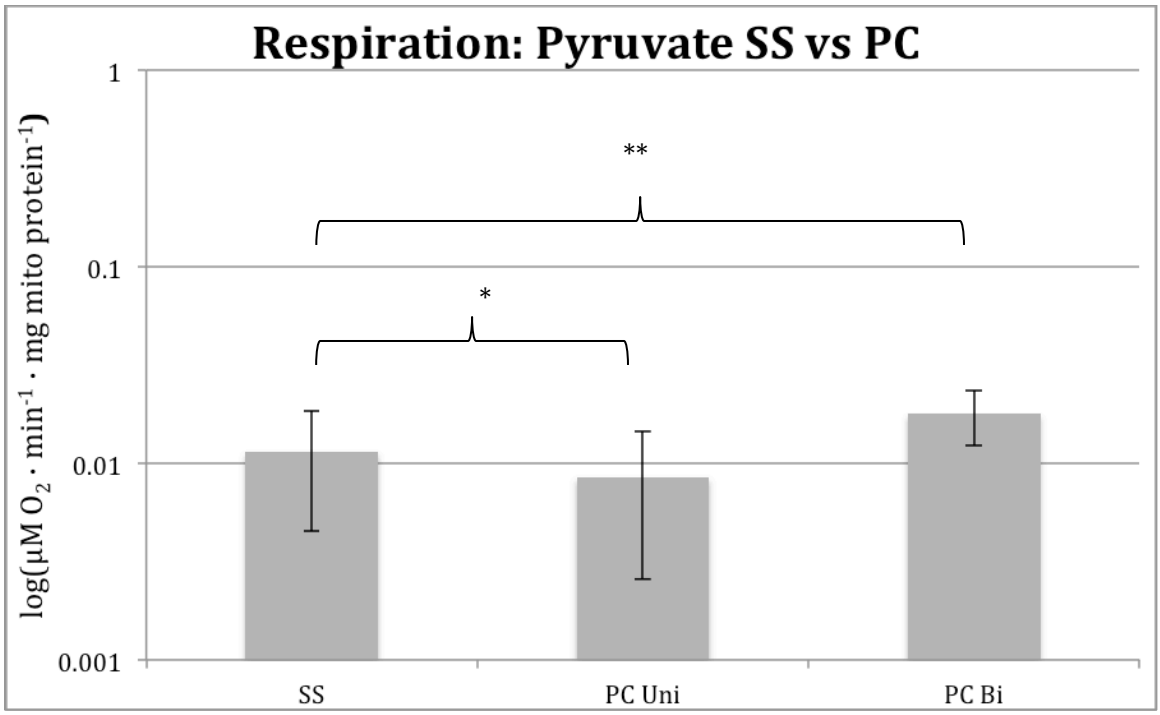

Figure 15: Comparison of respiration rate of Sham Sham (SS) group compared to the two preconditioned groups for substrate pyruvate on a logarithmic scale.

Preconditioned, unilaterally injured rat kidney cortex mitochondria fractions (PC Uni) and preconditioned, bilaterally injured rat kidney cortex mitochondria fractions (PC Bi). There are no differences between the SS group and PC Uni group (*, $p>0.05$), and there is a difference between the SS group and PC Bi group (**, $p<0.05$). Oxygen consumption is measured in micromole oxygen consumed per minute, per milligram of mitochondria protein (as determined from the Bradford Assay). SS n=27, PC Uni n=12, PC Bi n=10.

Using pyruvate as a substrate, after IRI, mitochondria respiration of PC Uni mitochondria is respiring no different from SS mitochondria ($p>0.05$) and PC Bi mitochondria are performing *better* than SS mitochondria ($p<0.05$).

3.1.1 Sham Sham rat renal mitochondria respiration rate is similar to vehicle injected without IRI rat renal mitochondria only for one substrate succinate

For the substrate succinate, SS mitochondria respiration rate is similar to that of vehicle (saline) injected mitochondria (without IRI) ($p>0.05$). For the other two substrates, isocitrate and pyruvate, respiration rates of VwoI mitochondria are significantly depressed compared to SS mitochondria ($p<0.05$). With SS mitochondria as a baseline control group,

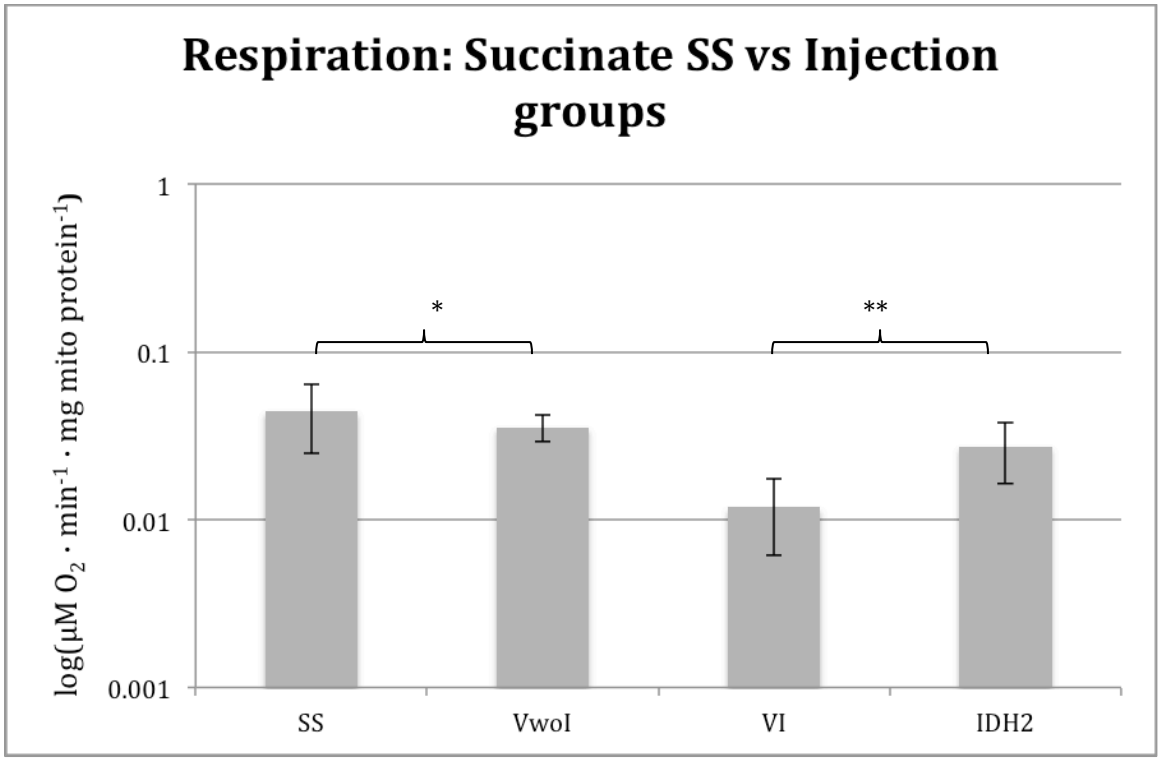

Figure 16: Comparison of respiration rates of Sham Sham (SS) group compared to all the injection groups, vehicle without injury, vehicle with injury and IDH2 for the substrate Succinate on a logarithmic scale.

Oxygen consumption is measured in micromole oxygen consumed per minute, per milligram of mitochondria protein (as determined from the Bradford Assay). * - No difference between SS and VwoI groups. ** - Significant difference between IDH2 and VI groups, from Shijun Zhang's thesis. SS n=27, VwoI n=9, VI n=9, IDH2 n=9.

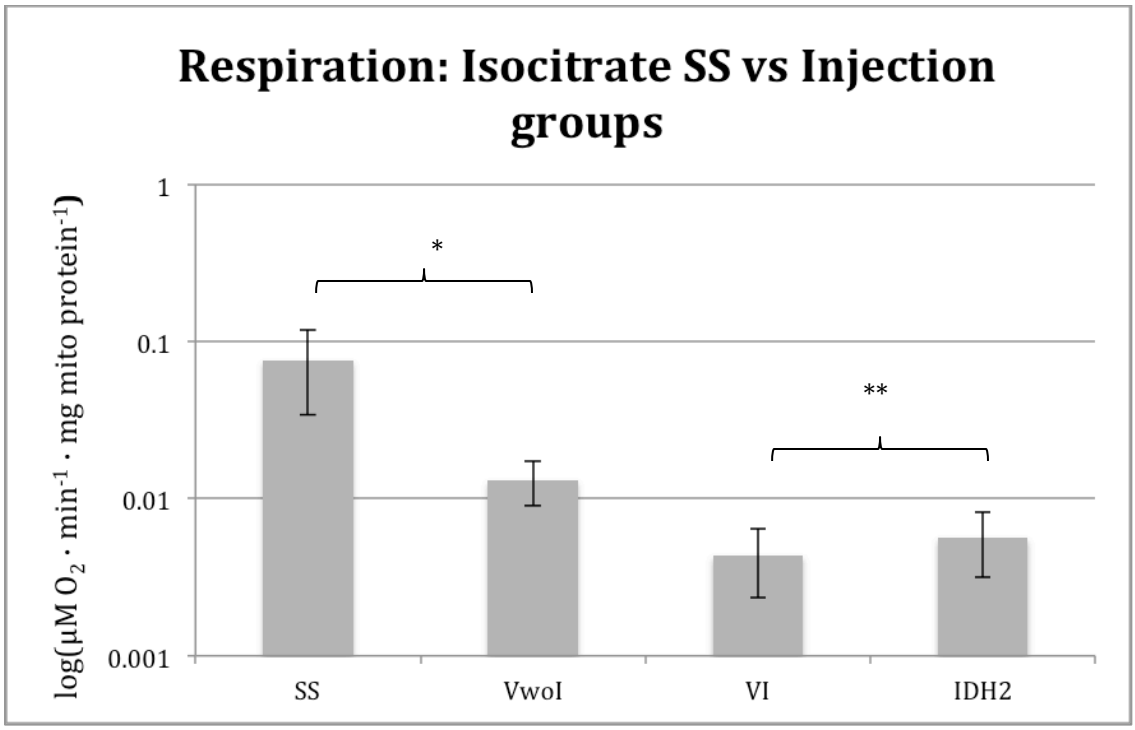

Figure 17: Comparison of respiration rates of Sham Sham (SS) group compared to all the injection groups, vehicle without injury, vehicle with injury and IDH2 for the substrate Isocitrate on a logarithmic scale.

Oxygen consumption is measured in micromole oxygen consumed per minute, per milligram of mitochondria protein (as determined from the Bradford Assay). * - Significant difference between SS and VwoI groups. ** - Significant difference between VI and IDH2 groups (from Shijun Zhang's thesis).. SS n=27, VwoI n=9, VI n=9, IDH2 n=9.

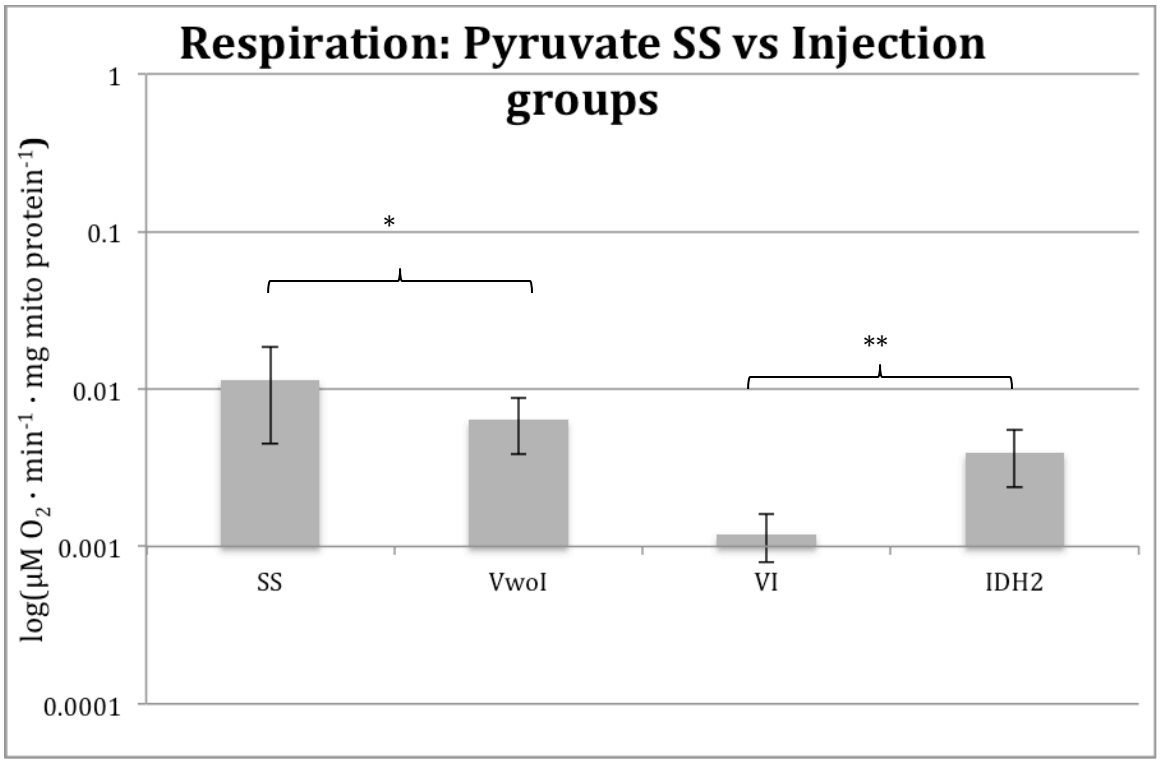

Figure 18: Comparison of respiration rates of Sham Sham (SS) group compared to all the injection groups, vehicle without injury, vehicle with injury and IDH2 for the substrate Pyruvate on a logarithmic scale.

Oxygen consumption is measured in micromole oxygen consumed per minute, per milligram of mitochondria protein (as determined from the Bradford Assay). * - Significant difference between SS and VwoI groups. ** - Significant difference between VI and IDH2 groups (from Shijun Zhang's thesis). SS n=27, VwoI n=9, VI n=9, IDH2 n=9.

3.1.3 EGTA as an ingredient in Terzic buffer allows for improved respiration as compared to EDTA

In the buffer used for the respiration chamber, we used EDTA as an ingredient but a review of the protocol revealed that EGTA could possibly be a better ingredient. Both EDTA and EGTA chelate for both calcium and magnesium, but EDTA has a higher affinity for chelating magnesium, an important cofactor for the proper function of isocitrate dehydrogenase 2, our protein of interest. EGTA has a lower affinity for magnesium and a high affinity for calcium. Our results revealed that there is a significant difference in respiration rates for EDTA compared to EGTA buffers for the substrate succinate but a negligible difference for respiration for the substrates isocitrate and pyruvate.

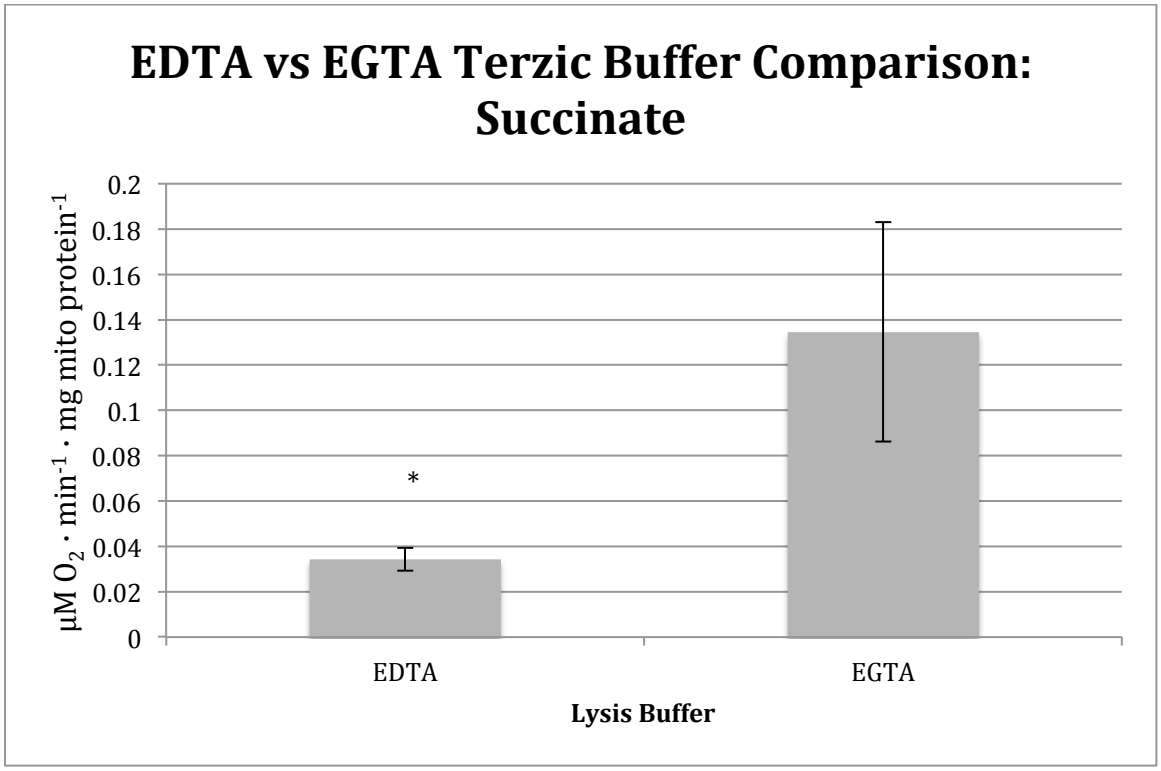

Figure 19: Comparison of EDTA vs. EGTA as an ingredient in Terzic buffer for the substrate Succinate.

EGTA used in the terzic buffer resulted in a significantly improved respiration rate (*, $p<0.05$) compared to terzic made with EDTA. N=6 (3 right kidneys and 3 left kidneys).

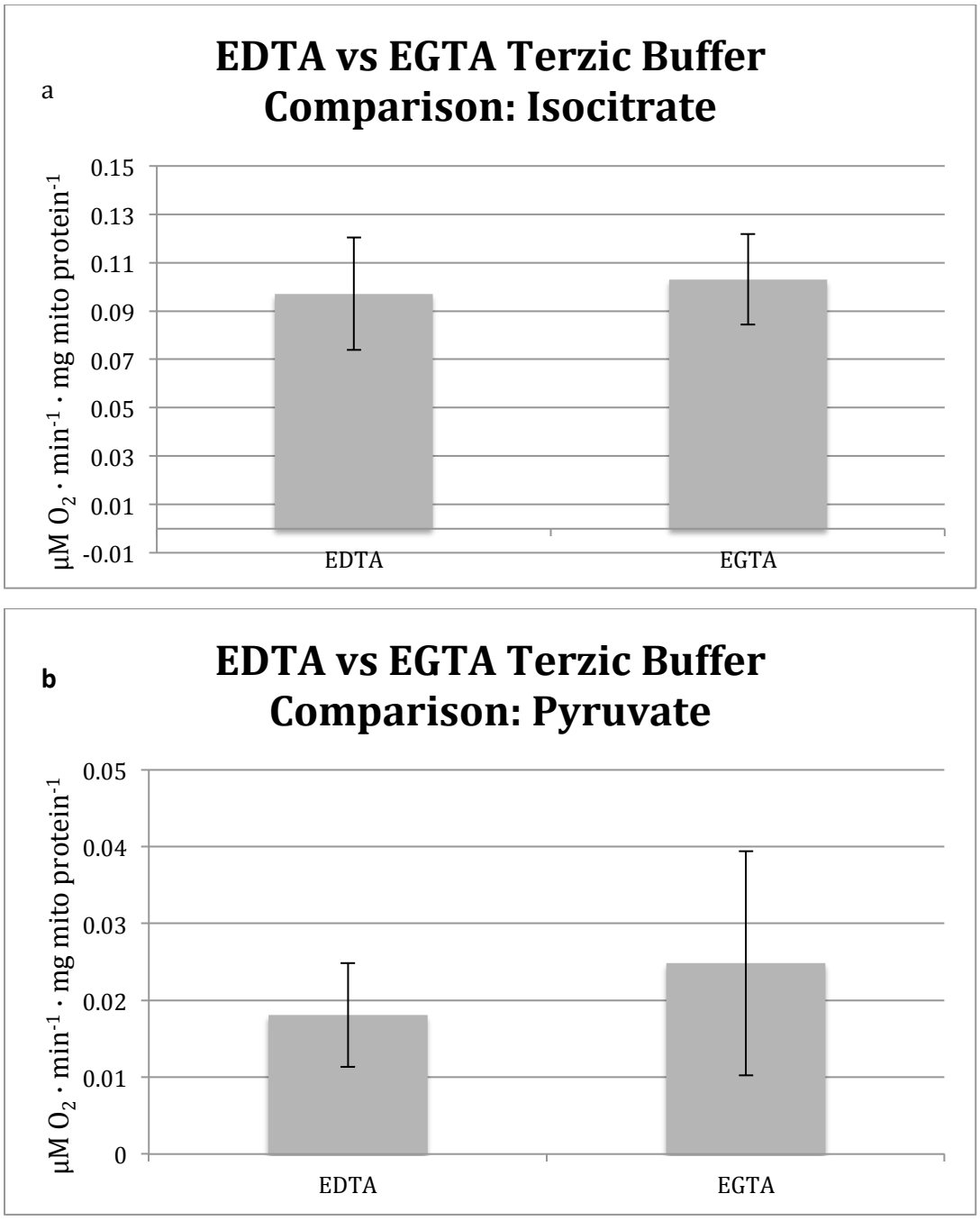

Figure 20: Comparison of EDTA vs. EGTA as an ingredient in Terzic buffer for the substrates Isocitrate (a) and Pyruvate (b).

EGTA used in the terzic buffer resulted in similar respiration rates ($p<0.05$) compared to terzic made with EDTA for both isocitrate and pyruvate. N=6 (3 right kidneys and 3 left kidneys).

3.2 Western blot analysis of IDH2 protein in Preconditioned samples shows elevated IDH2 levels in Sham injury bilateral (SI Bi), Preconditioned Unilaterally injured (PC Uni) and Sham unilaterally injured (SI Uni) samples is markedly decreased compared to controls.

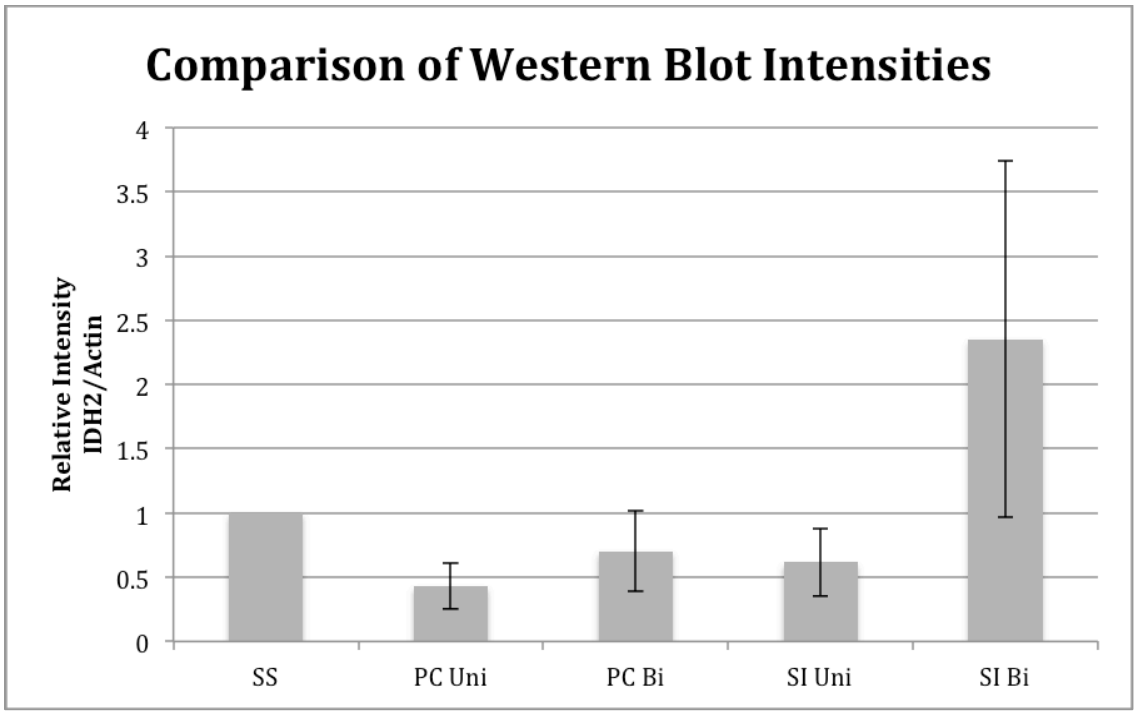

Figure 21: Quantification of IDH2 protein expression in Preconditioned study groups via Western blot analysis.

The amount of IDH2 in Preconditioned rat kidney groups were quantified and compared to the control group (SS). Analyses of band intensity on films are presented as a ratio relative to actin intensities.

Western blot analysis on IDH2 versus Actin fluorescent intensities showed that the levels of IDH2 proteins are depressed in PC Uni, PC Bi and SI Uni groups while SI Bi group has increased IDH2 protein. The increase or decrease in IDH2 protein levels show that protein levels change within an hour.

3.3 Hydrodynamically Delivered Mitochondrial Protein IDH2 Protects Rat Kidney Mitochondria against Moderate Ischemic/Reperfusion Injury

In this procedure, there were three groups of rats: two of which received hydrodynamic delivery of saline only (VwoI and VI), and one of which received hydrodynamic delivery of IDH2 plasmid (IDH2). At 7 days post injection, one of the vehicle groups (VI) and the IDH2 group received a 40-minute unilateral ischemia to the same kidney that was injected with the respective solution, followed by a 1-hour reperfusion. The last group (VwoI) was sacrificed without injury. Kidney tissues were harvested from all three groups and the mitochondrial fractions isolated from the kidney tissue. Mitochondrial fractions were then used in the mitochondrial oxygen consumption assay.

After ischemia reperfusion injury (IRI), mitochondrial oxygen consumption of each fraction in state III was measured based on the Chance and Wooden protocol[170]. The results of the oxygen consumption assay are shown. For the substrate succinate, mitochondrial oxygen consumption of IDH2 injected rats (IDH2) had no significant difference from vehicle rats without injury (VWOI), $p>0.05$, but significantly different, $p<0.05$, from vehicle with injury group (VI). IDH2 group's oxygen consumption remains slightly depressed from the VWOI group.

For the substrate pyruvate, mitochondrial oxygen consumption of IDH2 injected rats (IDH2) receiving IRI had no significant difference from vehicle rats without injury (VWOI), $p>0.05$, but significantly different, $p<0.05$, from vehicle with injury group (VI). IDH2 group's oxygen consumption remains slightly depressed from the VWOI group. The upregulation of IDH2 in IDH2 injected rats is confirmed via western blot analysis, with a significant increase of IDH2 in IDH2 injected rats compared to VWOI and VI rats.

Thus, upregulation of IDH2 in hydrodynamically gene delivered kidneys resulted in improved mitochondrial oxygen consumption compared to VI or injured rats.

Mitochondria oxygen consumption was significantly impaired after IRI injury, but the overexpression of IDH2 genes ameliorated the decrease in oxygen consumption. This protective effect is analogous to the protective effect of preconditioning observed in previous studies comparing serum creatinine values.

3.3.1 Overexpression of transgene confirmed via western blot analysis

In total, there were six groups of rat kidney cortex samples. One group, sham (SS) rats underwent sham surgery at both day 0 and day 14, sham injury rats underwent sham surgery at day 0 and IRI surgery at day 14, preconditioned rats (PC) underwent IRI surgery at both day 0 and day 14, vehicle without injury rats underwent hydrodynamically injected saline injection at day 0 and sham surgery at day 7, vehicle with injury rats underwent hydrodynamically injected saline injection at day 0 and IRI surgery at day 7 while IDH2 rats underwent hydrodynamically injected IDH2 injection at day 0 and IRI surgery at day 7. There were increased levels of IDH2 expression in rats that received hydrodynamic delivery of the IDH2 plasmids, compared to the vehicle rats. By densitometry analysis of the western blot, IDH2 expression in the IDH2 delivered rats was 2.2 times higher than VWOI rats. The IDH2 expression of PC rats were 2.8 times higher than SS rats, only for bilaterally injured animals and 1.6 times higher than SS rats for unilaterally injured animals. The western blot analysis shows that there is upregulation of IDH2 enzymes through hydrodynamic injection and through bilateral ischemic preconditioning.

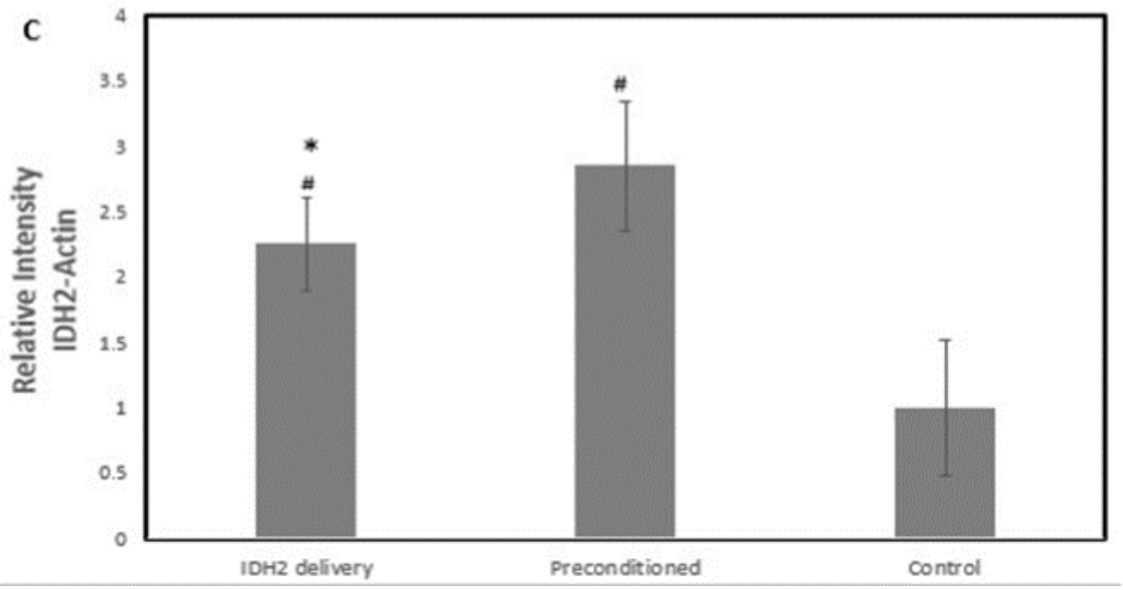

Figure 22: Quantification of IDH2 protein expression in rat kidneys via Western blot analysis.

The amount of IDH2 in IDH2 delivery rat kidneys and Preconditioned rat kidneys were quantified and compared to the control group (saline injected). Analyses of band intensity on films are presented as a ratio relative to actin intensities. *P>0.05 compared to Preconditioned. #P<0.05 compared to Control (saline injected). Taken from Shijun Zhang's thesis [172]

3.4 Morphology of each group of mitochondria under Electron Microscopy

While preparing the kidney cortex samples for mitochondria purification, we saved samples of the cortex for transmission electron microscopy in order to visualize changes in mitochondria morphology in control and treatment groups. Normal mitochondria are characterized by regular shape, dense matrix and parallel cristae[173]. Mitochondria injury can be visualized by changes in shape, number or size of mitochondria, changes in density of matrix and increased space among cristae, or swelling of the mitochondria. Through TEM, we are observing the degree of injury in each treatment group and the associated morphological changes associated with each treatment, which can allow us to speculate on possible mechanisms behind mitochondrial adaptations to injury.

The following images are preliminary images from a few groups in the preconditioned and injection study groups, and thus conclusions cannot yet be drawn about the relationship between each treatment and the mitochondrial morphological changes associated.

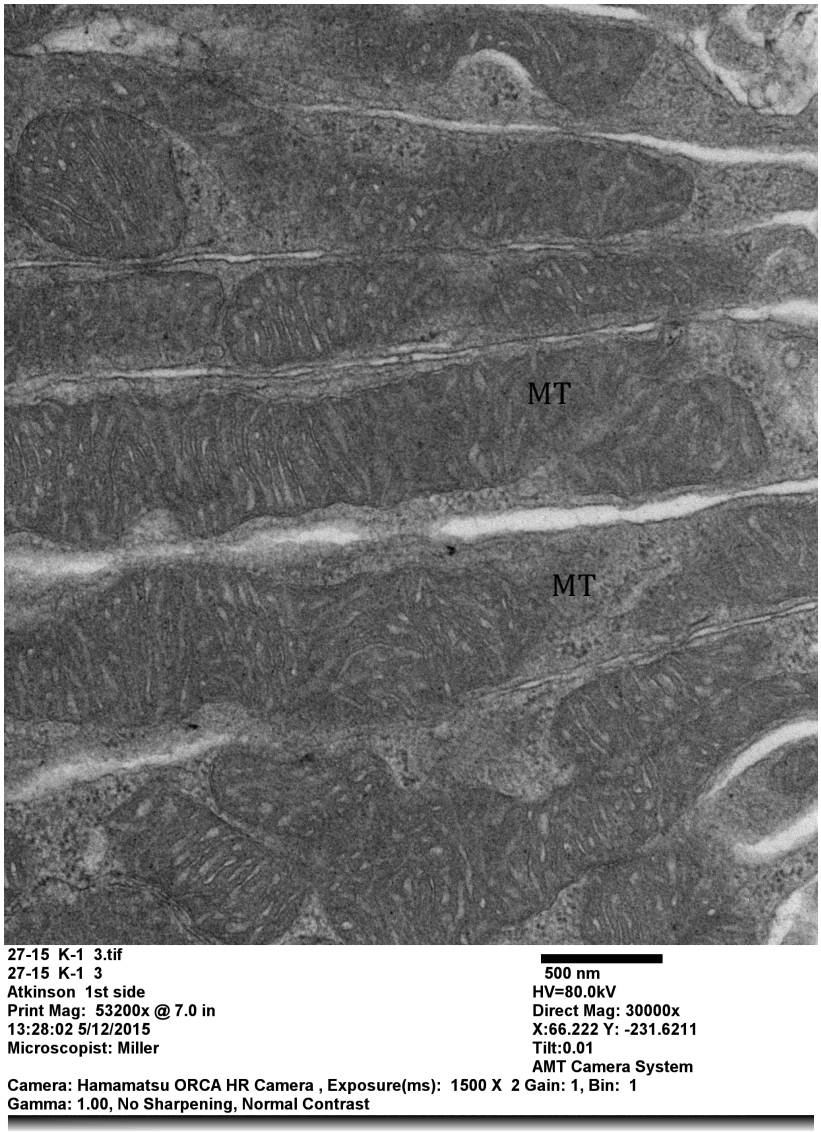

Figure 23: TEM image of Sham Sham rat kidney cortex.

The mitochondria of the sham sham samples appear normal, with regular shape, dense matrix and parallel cristae, hallmarks of healthy mitochondria[173]. MT – Mitochondria.

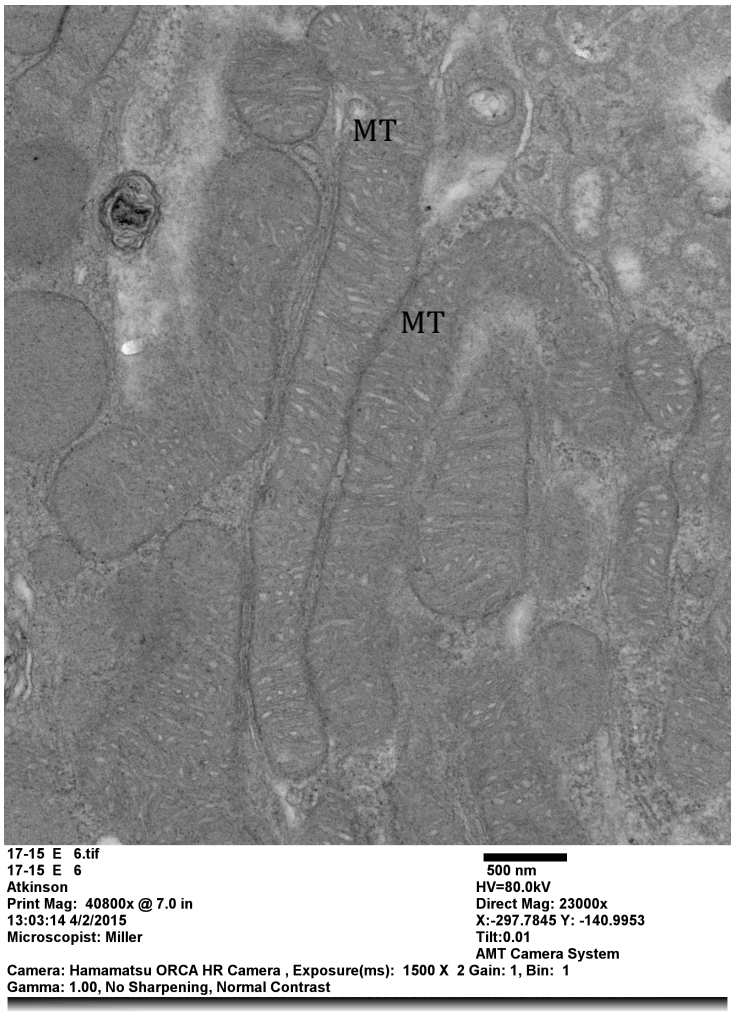

Figure 24: TEM image of PC Uni rat kidney cortex.

Mitochondria appear generally regular, with more healthy appearing mitochondria than SI Uni images. MT – Mitochondria.

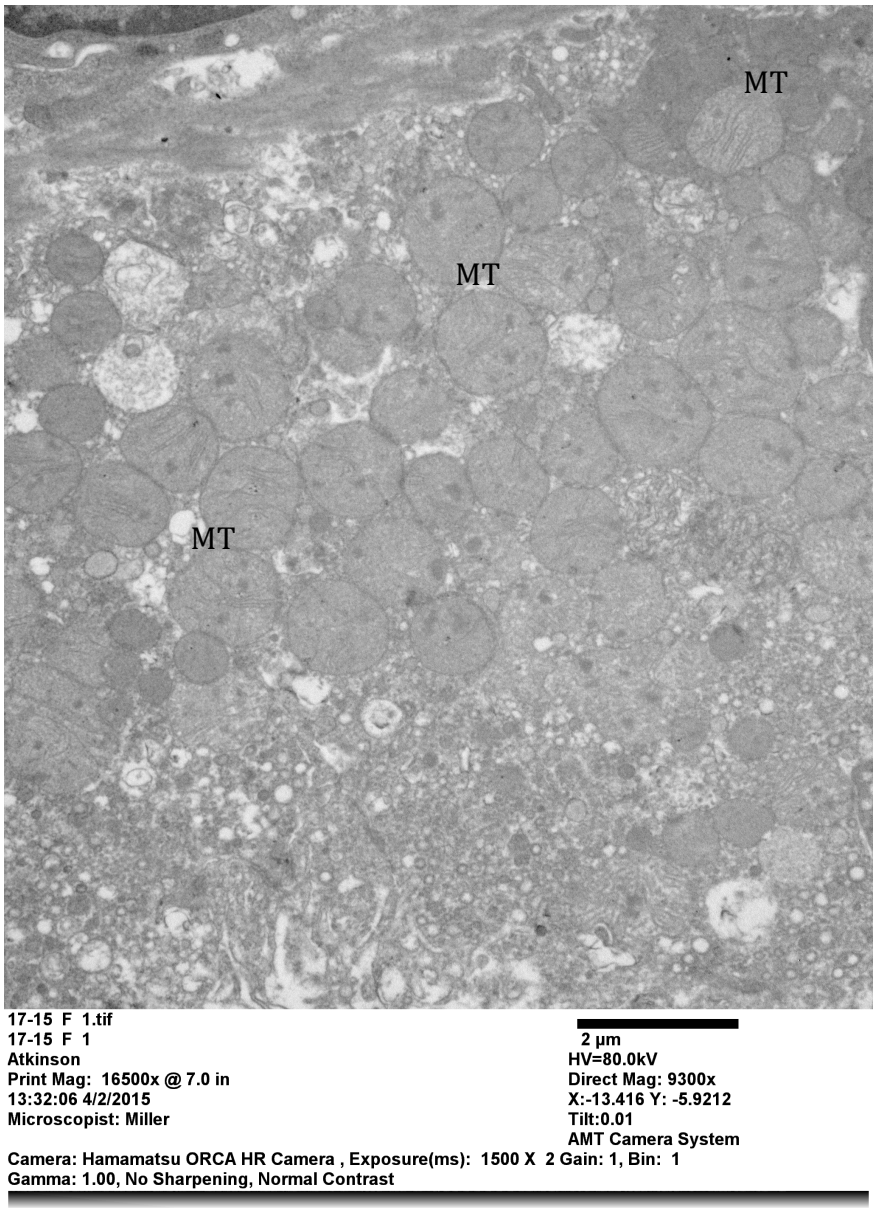

Figure 25: TEM image of SI Uni rat kidney cortex.

There is a lack of brush borders and not many recognizable structures. Morphology is drastically altered, with a lack of cristae in the mitochondria. Proximal and distal tubules could not be distinguished from one another. MT – Mitochondria

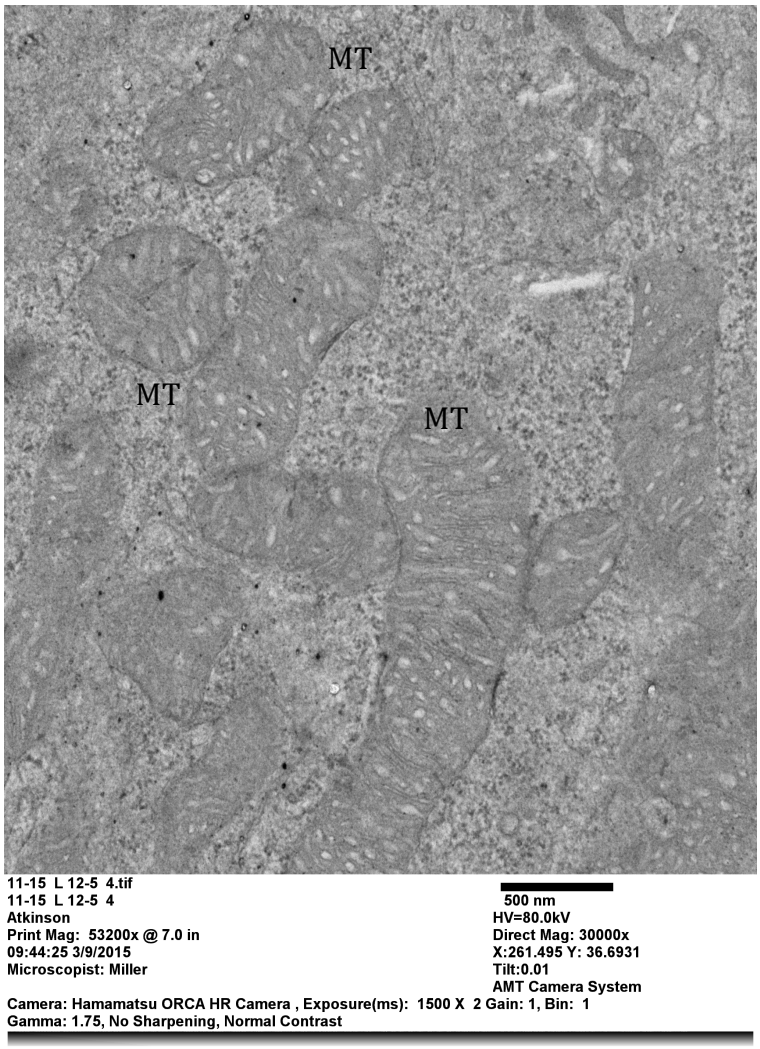

Figure 26: TEM image of Vehicle without injury rat kidney cortex.

Mitochondria appear swollen with spaces in between the cristae. MT – Mitochondria.

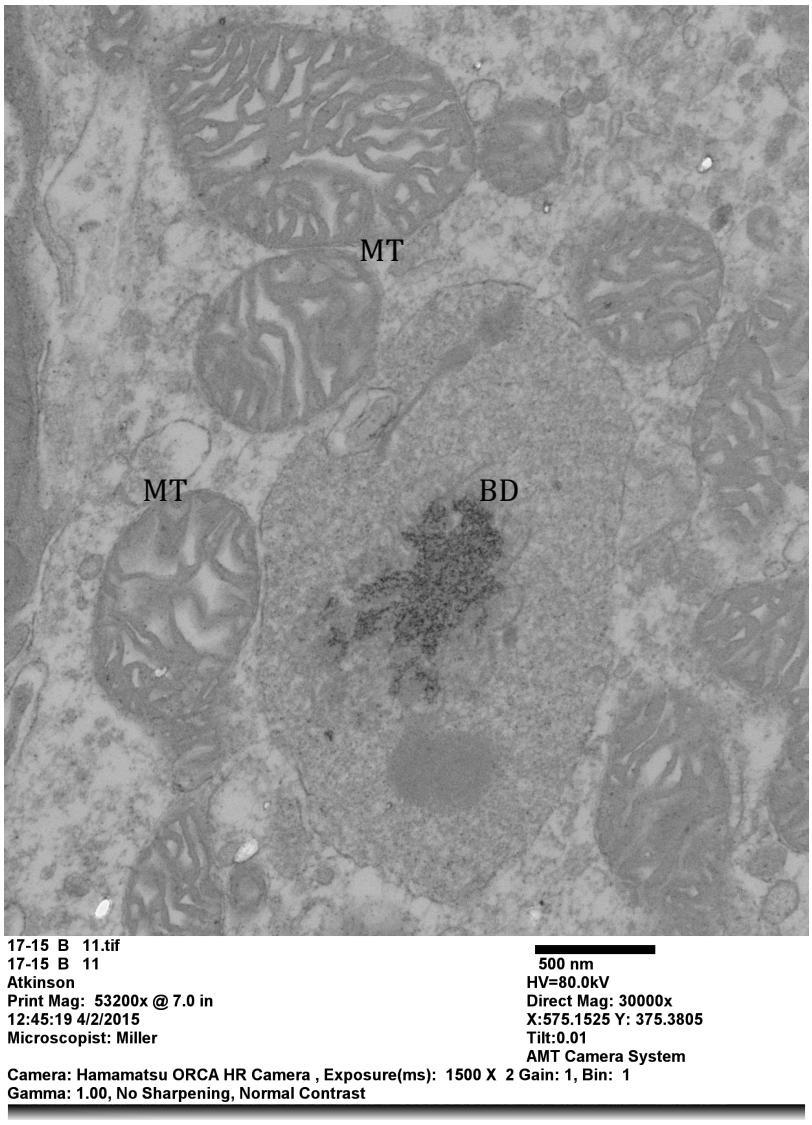

Figure 27: TEM image of IDH2 injected rat kidney cortex.

Mitochondria appear significantly more swollen than vehicle injected rats with abnormal cristae. Unidentified black deposits also appear throughout the sample. MT – Mitochondria.

CHAPTER 4. DISCUSSION

4.1 Summary of Results

In previous studies performed by Dr. Peter Corridon in the Atkinson lab, the efficiency of hydrodynamic plasmid delivery was tested using fluorescent plasmid vectors to the rat kidney. In ischemic preconditioning studies, the mitochondrial proteins IDH2 and SULT1C2 were identified as upregulated in preconditioned without second injury rats as compared to sham rats, and preconditioning conferred a resistance to kidneys from subsequent insult. The delivery of eGFP, IDH2 and SULT1C2 were confirmed via confocal and two photon imaging (eGFP) or western blot (IDH2 and SULT1C2), showing that the hydrodynamic fluid injections facilitated the delivery of transgene to target cells in the rat kidney. Both preconditioned and IDH2 plasmid injected rats demonstrated resistance to further insults in the form of IRI, as shown in serum creatinine measurements.

Thus, we are trying to discern the function of the preconditioning effect via injection of IDH2 and mitochondria oxygen consumption in both preconditioned and IDH2 rats. Since IDH2 protein is upregulated in a preconditioned without second injury kidney as compared to a control, conferring protection onto the kidney against subsequent IRI as seen in serum creatinine values, we performed in vitro assays of mitochondria oxygen consumption on IDH2 injected and preconditioned kidneys. As an essential enzyme in the citric acid cycle, IDH2 functions to modify mitochondria oxygen consumption.

All mitochondria respiration data has been displayed on a logarithmic scale, as there is large variability when our respiration values are large, and smaller variability when our respiration values are smaller, which corresponds to data fitting a logarithmic scale.

4.2 The Implications of ischemia reperfusions injuries on rat kidneys and its effect on mitochondria respiration.

Our hypothesis is that upregulated expression of IDH2, either through preconditioning, or hydrodynamically delivered exogenous IDH2 plasmids, will protect renal function from subsequent insults by enhancing mitochondrial respiration. The increased expression of IDH2 may enhance certain pathways involved in mitochondria respiration such as the TCA cycle, electron transport chain and oxidative phosphorylation, and improve or maintain cell function against AKI. In our studies, the two pronged effort to perform mitochondria oxygen consumption assays on preconditioned groups and injection groups can help us discern the pathway through which the preconditioning confers protection and use the knowledge derived from these experiments to mimic the protective effect of preconditioning, while avoiding the unacceptable clinical risks of ischemia reperfusion.

According to our data from the comparison of control group to our sham unilateral injury group (SI Uni), the insult to the left kidney resulted in depressed mitochondria respiration rates and decreased levels of IDH2 in the left kidney according to western blot (the right kidney data is not included). In contrast, according to our data comparing control group to sham bilateral injury group (SI Bi), for all three substrates, bilateral injury resulted in increased mitochondria respiration rates and increased levels of IDH2 according to western blot analysis. This is consistent with increased levels of IDH2 contributing to increased respiration rates.

The different effects of unilateral versus bilateral injury on both respiration and IDH2 levels are surprising. Unilateral ischemic injury (without preconditioning) results in depressed mitochondria respiration rates but, if there is bilateral ischemic injury, the recovery process in the mitochondria during reperfusion is drastically different from when only one kidney is injured. When only one kidney is injured, there could be neurohormonal mechanisms in place that signal to contralateral kidney to compensate for the ischemic insult to the left kidney. In septic sheep kidneys, blood flow to the contralateral kidney is significantly elevated resulting from increased sympathetic nervous response in both the heart and the kidneys[79].

The SI Bi group tells a story different from SI Uni group about the injury process when the renal system is entirely impaired. Mitochondria respiration rates for SI Bi are significantly higher than the control group, with correlating higher IDH2 protein levels as well. It suggests that when both kidneys are injured, during reperfusion, there are processes at work during respiration that ramp up respiration rates, possibly through the increased IDH2 protein levels, which could be the driving force behind the increased runs of TCA cycle.

When only one kidney is injured, during reperfusion there could be processes at work that allows for a different recovery process; the mitochondria might be diverting resources to re-equilibrating membrane potentials, restoring proton and ion concentrations across membranes, repairing electron transport chain complexes, or metabolizing the build up of ROS[93, 110-112, 116, 117]. Or, due to diversion of blood flow to the contralateral kidney from sympathetic nervous system responses, the decreased blood flow to the left kidney could further impair the recovery process in the left kidney, resulting in significantly depressed respiration rates in the SI Uni kidney. Although a reliable conclusion cannot be drawn yet from the electron microscopy images, the drastic damage to SI Uni mitochondria suggests that it is probably due to the later situation, where blood flow is diverted to the right kidney during the recovery

process. In SI Bi kidneys, there could be a more equal divide in blood flow during reperfusion as the system detects that both kidneys are injured, resulting in improved recovery in the reperfusion period. Also, with a 40 minute ischemia and 1 hour reperfusion, the 1 hour reperfusion time shows the quick elevation in IDH2 protein levels and thus shows the quick response in the preconditioning event.

A literature review revealed that many ischemic reperfusion studies are usually done bilaterally [71, 75, 79, 90, 174-176]or unilaterally with a previous nephrectomy on the right kidney[177, 178]. In studies where unilateral injuries are performed without a contralateral nephrectomy, contralateral kidney data are generally grouped with sham controls, with no notable comment on distinctions found between them[58].

4.3 The Implications of preconditioning on rat kidneys and its effect on mitochondria respiration.

According to our data, most of the preconditioned and subsequently unilaterally or bilaterally injured rat renal mitochondria (PC Bi or PC Uni) respire at a rate similar ($p>0.05$) or better (pyruvate: SS versus PC Bi, $p<0.05$) to our control group (SS) for two substrates, succinate and pyruvate, but not for isocitrate: SS versus PC Uni ($p<0.05$). This trend is similar to our findings from the injection groups, where IDH2 injected and subsequently injured renal mitochondria respires at a similar rate ($p>0.05$) to the saline delivered, non-injured group. The findings of generally non-depressed mitochondria respiration rates of PC Uni and PC bi groups support the previous findings, when serum creatinine values of PC rats remain low even after a second insult compared to rats which have not undergone preconditioning, and shows that the protection conferred by preconditioning extends to mitochondrial function.

However, the western blot analysis of IDH2 protein levels show depressed levels of IDH2 for both PC Uni and PC Bi groups compared to controls, and similar to levels of

SI Uni groups. This result differs from our expectation that IDH2 levels would correlate with mitochondrial respiration, and suggests that preconditioning may be able to confer protection on mitochondrial function by other mechanism.

If we were to take each treatment group and amalgamate the data into one timeline, there are some conclusions to be drawn about the level of respiration and the level of IDH2 level. It is particularly notable that there are distinct responses in terms of respiratory rate and IDH2 levels to an initial bilateral injury versus a second injury to the same kidney. Following the first IRI event, as illustrated in the SI Bi group, after one hour reperfusion subsequent to a moderate ischemic injury, both kidneys are respiring at higher rates than controls and IDH2 protein levels are increased. The increased IDH2 protein levels could be possibly due to increased gene expression in response to the injury, and rat renal mitochondria is ramping up respiration to compensate for the injury.

In the days after the first IRI, or the preconditioning event, both kidneys recover as serum creatinine levels return to baseline levels. When there is a second IRI, represented by the values from the PC Bi group, and in contrast to the events after the first injury, IDH2 protein levels are depressed, but the mitochondria have adapted to the injury and are possibly able to respire at higher efficiency than previously due to the adaptations from the first IRI event, but without the assistance of increased IDH2 levels. With depressed IDH2 levels, there could be fewer cycles of the TCA cycles but each electron carrier and electron carried through the ETC could be carried more efficiently. For the substrates isocitrate and succinate, PC Bi respired at a level similar to SS controls but pyruvate respired significantly better than SS controls. Since pyruvate is involved at the beginning of the TCA cycle as a 3-carbon precursor, increased pyruvate concentrations in the respiration chamber could be driving force behind pushing the TCA cycle forward.

4.4 Hydrodynamic fluid injections of IDH2 plasmids partially mimics the preconditioning effect

Our data from hydrodynamic fluid injections of IDH2 plasmids, obtained from Shijun Zhang's thesis[172], shows improved mitochondria respiration rates for IDH2 groups compared to vehicle injury groups (VI) for the substrates of pyruvate and succinate ($p<0.05$) but not for isocitrate. The IDH2 injection groups also show similar levels of respiration compared to the vehicle without injury control groups (VwoI). Levels of IDH2 protein in the IDH2 injection group are elevated post-IRI according to western blot analysis. Comparison of the injection groups to the preconditioning groups can yield information on the mechanism of preconditioning, recovery from an IRI and the hydrodynamic injection treatment.

The vehicle with injury group showed significantly depressed mitochondria respiration rates, which is reasonable considering there could be minor injury to the kidney following the injection (previous studies found that the injury is not significant with normal serum creatinine values), and a moderate IRI prior to sacrifice. IDH2 injected mitochondria showed significantly improved respiration rates, showing that the hydrodynamic injections of IDH2 plasmids do protect the kidney from IRI. The SI Uni data points are also allowing us to glean information about our injection models, as the right kidneys are always shams, even in a VI and IDH2 groups. When we compare the IDH2 group, which is unilaterally injured 7 days after a hydrodynamic fluid injection, to the PC uni and SI Uni across substrates, the IDH2 group respires more similar to the SI uni group than the PC uni group.

However, the IDH2 injected group has elevated IDH2 levels similar to the SI Bi group, which shows that there could be some similarity between those groups in terms of protein expression. And since mitochondria respiration levels are attenuated in the IDH2 injected kidneys after IRI, to the level of vehicle controls but not to the level of sham controls, there is some level of protection in the IDH2 injected kidneys conferred

by the injection. The level of protection is not yet up to the sham controls, and thus there is only partial protection conferred by the injection.

The TEM images of the various preconditioning study and injection groups show the various morphological changes that happen when the kidney is manipulated. The SI Uni image shows one extreme end of injury, which correlates with the depressed mitochondria respiration rates, with the loss of brush border and drastic morphological changes. The preconditioned kidneys, even with two injuries within two weeks, has more normal looking mitochondria than the SI Uni samples, which also corroborates with the normal mitochondria respiration rates, however it is still morphologically distinct from control SS kidney cortexes. The injection groups show that injections result in swollen mitochondria as seen in the enlarged spaces in between cristae, which is morphologically distinct from the SI Uni and PC Uni samples, suggesting an injury that is distinct from the preconditioning injury groups.

4.5 The mitochondria respiration assay can be further improved to provide more reliable and precise results

In our buffer comparison, EGTA as an ingredient in Terzic buffer for the mitochondria respiration assay in place of EDTA lead to improved respiration rates for the substrate succinate, and to a lesser extent, pyruvate. EGTA has more specific calcium-chelating properties and is also less specific for Mg^{2+} ions, an important cofactor in the TCA cycle, and this could be the reason for improved mitochondria respiration.

Another improvement to the mitochondria respiration assay is reduced quantities of substrate and ADP added to the mitochondria respiration chamber. We are unable to calculate a respiration control index (RCI), which is a ratio of state III (oxygen respiration) to state IV respiration (state of ADP depletion). The ratio represents a quantification of mitochondria coupling and the higher the ratio, the tighter the

coupling between the mitochondrial respiratory chain and oxidative phosphorylation, and is thus an important quantification of the quality of the respiration assay and purification process. In our studies, the chamber ran out of oxygen before it could reach state IV, a state of ADP depletion, and thus we could not calculate RCI.

CHAPTER 5. CONCLUSIONS

First, our lab performed studies to confirm that hydrodynamic fluid delivery could efficiently delivery with stable expression of plasmid vectors into live rodent kidneys. We used fluorescent plasmid EGFP-actin delivered by hydrodynamic fluid renal injection, and observed widespread expression with localization to cells of interest. The technique was fine-tuned through trial and error with various sites of injection before narrowing down to renal artery and vein clamps, with a renal vein injection to optimize delivery.

At the same time, preconditioning studies conducted by the Basile lab showed the preconditioning effect on the kidneys, where rats with preconditioned, or endured a first ischemic reperfusion injury, were protected from a subsequent injury, as evaluated by serum creatinine levels. Via proteomic analysis, we identified IDH2 and SULT1C2 as potential targets for hydrodynamic plasmid fluid delivery to the kidneys of live Sprague Dawley rats. In preliminary studies, via in situ PCR, amplified expression of the plasmids was observed in the tissue sections of kidneys and via immune blot, the overexpression of IDH2 and SULT1C2 through hydrodynamic plasmid delivery was also observed.

We then turned our attention to functional assays. Through TMRM fluorescent imaging, we observed that differences in mitochondria potential across preconditioned and hydrodynamic plasmid delivered rats, and also IDH2 and SULT1C2 are enzymes in the mitochondria, we performed mitochondria oxygen assays on preconditioned and

IDH2-injected rats to discern and elucidate the pathway through which the preconditioning effect is conferring protection against acute kidney injury.

In our studies with hydrodynamically IDH2 injected rats, we observed that the respiration rates for IDH2, injured kidneys are similar to vehicle injected, uninjured kidneys, showing that upregulated IDH2 does confer a protection on injured kidneys, similar to the preconditioning effect. The hydrodynamic fluid delivery of IDH2 can lessen the assault on mitochondria dysfunction done by a moderate acute kidney injury. Through mitochondria respirations with the substrates succinate and pyruvate, we observed no differences between IDH2 injured kidneys and vehicle uninjured kidneys. Interestingly, even though IDH2 is an isocitrate dehydrogenase, an important enzyme in the electron transport chain, renal samples in the mitochondria respiration assays ran with isocitrate showed no different between the IDH2 injured kidneys and vehicle injured kidneys, showing that the upregulation of IDH2 does not upregulate oxygen consumption in the mitochondria.

In our studies with preconditioned rats, we observed that the respiration rates of preconditioned, and subsequently injured, either bilaterally or unilaterally, are similar to those of sham kidneys. This supports the previous study, where serum creatinine was evaluated for the level of injury and protection. Interestingly, for our sham and subsequently injured bilaterally kidneys, the respiration rate was higher than the sham, which could provide a snapshot of the preconditioning effect, as the injury event is the first insult to the kidney. However, the respiration rate for sham and subsequently unilaterally kidneys was similar to vehicle injured (unilateral). Since the IDH2 injections are a unilateral injection and the subsequent injury to IDH2 injected or vehicle injected rats are unilateral, the depressed oxygen respiration rate for sham unilaterally injured rats and vehicle with second injury rates as compared to the normal oxygen respiration rate for IDH2 injected, injured rats show that IDH2 injections do confer protection onto the kidneys. The sham, bilaterally injured rats kidneys respire better than sham, which is

unexpected, but in our serum creatinine studies, serum creatinine is taken 24 hours post injury, whereas the mitochondria assay is performed 1 hour post injury and start of reperfusion, suggesting that during this one hour, there are mitochondria adaptations to injury, but during the 24 hour period, kidney function does suffer, and by day 14, when there is subsequent insult, kidney mitochondria has adapted and can endure a subsequent insult without sacrificing function.

In conclusion, our studies have demonstrated efficient and effect hydrodynamic fluid delivery of IDH2 plasmids, which can partially mimic the preconditioning effect although there can be no conclusions drawn yet as to whether it is the exact same pathway. Further studies are needed to improve our mitochondria respiration assays, investigate further the specific mechanisms of preconditioning beyond serum creatinine and oxygen respiration rates.

CHAPTER 6. FURTHER STUDIES

There are numerous ways in which the data obtained in this study can be fine-tuned. The Oroborus and Seahorse machines are dramatically more precise and advanced pieces of equipment that could hypothetically reduce the large error bars in the data. The use of EGTA as an ingredient in the Terzic buffer can be another change as well that will magnify the respiration rates of samples. We will continue to examine the mitochondrial oxygen consumption study in preconditioning groups of Sprague Dawley rats as well as hydrodynamic fluid delivery groups. Also, in the more advanced machines, a respiratory control ratio (ratio of State III respiration to State IV respiration) can be calculated.

Our mitochondria purification can also be modified according to Shalbuyeva and Brutoveksky[179]. A looser fitting homogenizer can result in a higher yield of mitochondria from the purification process.

Our data shows that IDH2 delivery does elevate mitochondrial oxygen consumption, but not to the levels as shown in the early preconditioning phase, as represented by SI Bi with its one hour of reperfusion. We have previously identified another target of gene delivery, SULT1C2, and we could perform hydrodynamic injection studies on SULT1C2, or a combination of IDH2 and SULT1C2 to mimic the full effect of preconditioning. Also, in order to understand preconditioning, we still need to determine the role that IDH2 and SULT1C2 plays in the mitochondrial respiration pathway.

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